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Influence of "added" whey protein isolate on probiotic properties of yogurt culture bacteria and yogurt characteristics

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INFLUENCE OF “ADDED” WHEY PROTEIN ISOLATE ON PROBIOTIC PROPERTIES OF YOGURT CULTURE BACTERIA AND YOGURT CHARACTERISTICS

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With love to my family

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES x

ABSTRACT xii

CHAPTER 1: INTRODUCTION 1
1.1 Whey 1
1.1.1 Uses and Functionality of Whey Proteins in Industry 2
1.1.2 Health Properties of Whey Proteins 5
1.2 Yogurt 7
1.3 Lactic Acid Bacteria 8
1.3.1 Probiotics 8
1.3.2 Health Benefits of Culture and Probiotic Bacteria 9
1.4 Effect of Whey Proteins on Probiotic and Culture Bacteria 10
1.5 Whey Protein Concentrate in Yogurt Manufacture 14
1.6 Whey Protein Isolate in Yogurt Manufacture 14
1.7 Justification 17

CHAPTER 2: MATERIALS AND METHODS 20
2.1 Experimental Design 20
2.2 Yogurt Manufacture 21
2.3 Preparation of Media 23
2.3.1 Peptone water 23
2.3.2 Acid tolerance broths 23
2.3.3 Bile tolerance broths 23
2.3.4 Broths for enumeration of culture bacteria 24
2.3.5 *Lactobacilli* MRS agar 24
2.3.6 M17 agar 24
2.3.7 *Streptococcus thermophilus* agar 25
2.4 Microbiological Analysis 25
2.4.1 Acid tolerance procedure for pure culture bacteria 25
2.4.2 Acid tolerance procedure for culture bacteria in fat free plain yogurt 26
2.4.3 Bile tolerance procedure for pure culture bacteria 26
2.4.4 Bile tolerance procedure for culture bacteria in fat free plain yogurt 27
2.4.5 Enumeration of pure culture bacteria 28

2.4.6 Enumeration of culture bacteria in fat free plain yogurt 28
2.4.7 Coliform Counts 29
2.4.8 Protease activity 29
2.5 Analytical Procedures 30
2.5.1 pH 30
2.5.1 Titratable acidity 31
2.5.2 Apparent viscosity 31
2.5.3 Syneresis 31
2.6 Sensory study 32
2.7 Statistical analysis 32

CHAPTER 3: RESULTS AND DISCUSSION 34
SECTION 1: Pure Culture Bacteria 34
3.1 Acid Tolerance 34
3.1.1 *Streptococcus thermophilus* ST-M5 34
3.1.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 35
3.2 Bile Tolerance 38
3.2.1 *Streptococcus thermophilus* ST-M5 38
3.2.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 39
3.3 Growth 43
3.3.1 *Streptococcus thermophilus* ST-M5 43
3.3.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 44
3.4 Protease activity 47
3.4.1 *Streptococcus thermophilus* ST-M5 47
3.4.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 48
SECTION 2: Yogurt Analysis 51
3.5 Acid Tolerance 51
3.5.1 *Streptococcus thermophilus* ST-M5 51
3.5.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 52
3.6 Bile Tolerance 56
3.6.1 *Streptococcus thermophilus* ST-M5 56
3.6.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 57
3.7 Growth 60
3.7.1 *Streptococcus thermophilus* ST-M5 60
3.7.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 62
3.8 pH 64
3.9 Titratable Acidity (TA) 66
3.10 Apparent Viscosity 68
3.11 Syneresis 70
3.12 Sensory Study 71

CHAPTER 4: CONCLUSIONS 77

REFERENCES 79

APPENDIX A. CONSENT FORM FOR CONSUMER STUDY 88

APPENDIX B. QUESTIONNAIRE FOR CONSUMER STUDY 89

VITA 90

LIST OF TABLES

Table 1. Fat free plain set-type yogurt formulations..... 22

Table 2. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate under the influence of acidic broth. 35

Table 3. Least Square Means (Log CFU/mL) for acid tolerance of pure *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 120 minutes. 37

Table 4. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of acid..... 37

Table 5. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate with the influence of bile (oxgall). 41

Table 6. Least Square Means (Log CFU/mL) for bile tolerance of pure *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 5 hours. 42

Table 7. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of bile (oxgall)..... 42

Table 8. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate during 60 hours of incubation. 46

Table 9. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 60 hours. 46

Table 10. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 protease activity (absorbance) in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate during 24 hours of incubation. ... 49

Table 11. Least Square Means (Absorbance) for protease activity of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 24 hours. 50

Table 12. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 viable cell counts from fat free plain yogurt containing 0, 1, 2 and 3% w/v of added whey protein isolate under the influence of acidic broth. 53

Table 13. Least Square Means (Log CFU/mL) for acid tolerance of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt containing 0, 1, 2 and 3% w/v of added whey protein isolate over the incubation period of 120 minutes at pH 2. 55

Table 14. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of acid..... 55

Table 15. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/w of added whey protein isolate with the influence of bile (oxgall). 58

Table 16. Least Square Means (Log CFU/mL) for bile tolerance of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt containing 0, 1, 2 and 3% w/w of added whey protein isolate as influenced by bile (oxgall) over the incubation period of 5 hours..... 59

Table 17. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt as influenced by added whey protein isolate concentration in the presence of bile (oxgall). 59

Table 18. Probability > F Value (Pr > F) for fixed effects of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts, *Streptococcus thermophilus* ST-M5 counts, apparent viscosity, pH, titratable acidity and syneresis of yogurts containing 0, 1, 2 and 3% w/v of added whey protein isolate over storage period of 35 days..... 61

Table 19. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentrations. 62

Table 20. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 as influenced by the storage period of 35 days. 62

Table 21. Least Square Means (Log CFU/mL) growth of pure *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein over the incubation period of 12 hours. 64

Table 22. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration. 64

Table 23. Least Square Means for pH of yogurts as influenced by added whey protein isolate concentrations. 66

Table 24. Least Square Means for pH of yogurts as influenced by the storage period of 35 days. 66

Table 25. Least Square Means for Titratable Acidity (TA) of yogurts as influenced by added whey protein isolate concentrations over storage period of 35 days. 68

Table 26. Least Square Means for Apparent Viscosity of yogurts as influenced by added whey protein isolate concentrations. 69

Table 27. Least Square Means for Syneresis of yogurts as influenced by added whey protein isolate concentrations over storage period of 35 days. 71

Table 28. Probability > F Value (Pr > F) for fixed effect of sensory attributes of yogurts containing 0, 1, 2 and 3% w/v added whey protein isolate..... 73

Table 29. Means for sensory properties of yogurts as influenced by added whey protein isolate. 73

LIST OF FIGURES

Figure 1. Acid tolerance of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 120 minutes. 34

Figure 2. Acid tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 120 minutes. 36

Figure 3. Bile tolerance of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 5 hours. 38

Figure 4. Bile tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 5 hours 40

Figure 5. Growth of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 60 hours. 43

Figure 6. Growth of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 60 hours 44

Figure 7. Protease activity of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 24 hours. 47

Figure 8. Protease activity of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 60 hours. 48

Figure 9. Acid tolerance of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 120 minutes. 51

Figure 10. Acid tolerance of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 120 minutes. 52

Figure 11. Bile tolerance of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 5 hours. 56

Figure 12. Bile tolerance of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 5 hours. 57

Figure 13. Growth of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt as influenced by added whey protein isolate concentration over storage period of 35 days. 60

Figure 14. Growth of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt as influenced by added whey protein isolate concentration over storage period of 35 days. 63

Figure 15. pH of yogurts as influenced by added whey protein isolate levels over storage period of 35 days. 65

Figure 16. Titratable acidity (TA) of yogurts as influenced by added whey protein isolate levels over storage period of 35 days 67

Figure 17. Apparent Viscosity of yogurts as influenced by added whey protein isolate levels over storage period of 35 days. 69

Figure 18. Syneresis (whey loss) of yogurts as influenced by added whey protein isolate levels over storage period of 35 days. 70

Figure 19. Means for sensory attributes of blueberry yogurt as influenced by added whey protein isolate. 72

Figure 20. Frequency for acceptability of blueberry yogurt as influenced by whey protein isolate addition. 74

Figure 21. Frequency for purchase intent of blueberry yogurt as influenced by whey protein isolate addition. 74

Figure 22. Frequency for purchase intent of blueberry yogurt as influenced by whey protein isolate addition, if panelist were informed about the extra protein and probiotic benefit of product. 75

ABSTRACT

Consumers are becoming conscious of their diet, increasing protein intake and avoiding carbohydrates and fats. Whey proteins have branch chain amino acids responsible for muscle building. Whey protein isolate (WPI) contains more than 90% protein. The effect of incremental addition of WPI on probiotic characteristics of pure cultures and cultures in yogurt and yogurt characteristics are not known.

The hypothesis was that "added" WPI will influence the characteristics of yogurt culture bacteria in pure form and in yogurt. The objectives were: to determine the influence of added WPI on (1) acid and bile tolerance, growth and protease activity of pure cultures *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12, (2) growth, acid and bile tolerance of starter culture from manufactured plain yogurt, (3) the physico-chemical characteristics of yogurt over its shelf life and (4) the sensory attributes of yogurt. WPI was used at 0, 1, 2 and 3% w/v. Acid tolerance was conducted on pure cultures and cultures from manufactured plain yogurt at 30 minutes intervals for 2 hours of incubation and bile tolerance at 1 hour intervals for 5 hours. Yogurt was manufactured using 0 (control), 1, 2 and 3% WPI. For sensory evaluation, blueberry yogurt was manufactured using the same WPI concentrations. Physico-chemical analyses of yogurts were conducted every 7 days during 35 days of storage. Enumeration of yogurt cultures during yogurt´s shelf life was evaluated at 7, 21 and 35 days of storage. Sensory evaluation was conducted on yogurt 7 days after its manufacture. Data were analyzed using Proc Mixed model of SAS® 9.3 program and by analysis of variance (ANOVA) using Proc GLM. Significant differences between means were analyzed at $\alpha = 0.05$ using Tukey´s adjustment. Use of 2% WPI improved acid tolerance of *Streptococcus thermophilus* ST-M5 in yogurt. Use of 2 and 3% WPI improved bile tolerance of *Lactobacillus bulgaricus* LB-12 over the 5 hours of incubation.

WPI decreased syneresis of yogurts and improved sensory attributes of flavored yogurt. Overall liking scores were higher for 1% WPI yogurts compared to control. Overall, 1 or 2% WPI can be recommended in manufacture of higher whey protein yogurts.

CHAPTER 1: INTRODUCTION

1.1 Whey

Whey is a liquid by-product of cheese manufacture and represents up to 85% of total volume of milk (de Wit, 1998, Madureira *et. al*., 2007). It is rich in nutrients such as proteins, essential amino acids, lactose, salts and lipids (Siso, 1996, de Wit, 1998, Madureira *et. al*., 2007). These components are extracted from whey by physical or chemical separation techniques such as precipitation, filtration, dialysis or ion exchange (ADPI, 2002).

Using these industrial techniques, protein is separated from whey until non-protein material is extracted to acquire a specific concentration of protein (FDA, 2013). Different whey powders are available in market as whey proteins concentrates (25–89% protein), isolates (>90% protein) and hydrolysates which are enzymatically treated for rapid absorption (ADPI, 2002, Manninen, 2009). The major proteins in whey are β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and immunoglobulins (IG) and constitute 20% of total protein content of milk (Davis *et. al*., 2004, Hoffman and Falvo, 2004, Madureira *et. al*., 2007).

According with the Code of Federal Regulation 21 CFR 184.1979c (FDA, 2013), whey protein concentrate is extracted from liquid whey by precipitation or filtration. The finish dry product must contain not less than 25% of total protein. It can contain between fat (1-10%), ashes (2-15%), lactose (maximum of 60%) and moisture (up to 6%).

Whey protein isolate is a creamy-white powder extracted from whey by the process of liquid whey to remove non-protein components to obtain more than 90% of protein (ADPI, 2002).

Non-protein constituents are separated from liquid whey to obtain WPI by membrane filtration, precipitation or ion exchange. The typical composition of whey protein isolate is protein (>90%), fat (1%), lactose (0.5%), ashes (2%) and moisture (up to 4.5%) (ADPI, 2002).

1.1.1 Uses and Functionality of Whey Proteins in Industry

Whey proteins are commonly used in dietetic formulations and as ingredient in food industries such as bakery and dairy (de Wit, 1998). Common uses of whey proteins include protein supplementation, gelation of products (yogurts and pudding), water-binding (sausage and meat products) and emulsifier (ice cream, mayonnaise, margarine) (ADPI, 2002).

Whey protein fractions [β-lactoglobulin (β-LG)] were evaluated in frankfurter sausages and showed reduction of cook loss, increase of hardness and no detrimental sensory properties when β-LG (6.6%) was added compared to control (4% β-LG) (Hayes *et. al.*, 2005). Whey protein concentrate (WPC) was used in edible coating to extend shelf life of fresh cut apples and shown to be more effective in reducing browning along with some antioxidants that antioxidants by themselves (Perez-Gago *et. al*., 2006). Whey protein concentrates are used in the meat industry in reduced fat products. The use of WPC (35% protein) showed improvement in the water holding capacity and cook loss of reduced fat sausages compared with no addition of WPC in reduced fat sausages (Hughes *et. al*., 1997). In the same way, addition of WPC increased hardness, adhesiveness, gumminess and chewiness of a reduced fat sausage (Hughes *et. al*., 1997).

Whey protein concentrate (35% protein) was used in burger patties to evaluate physical and sensory properties (Desmond *et. al*., 1998). Addition of up to 2% of WPC reduced the shear force in low-fat beef burger patties compared with no addition of WPC (Desmond *et. al*., 1998). Addition of up to 3% of WPC increased hardness and chewiness of a low-fat burger compared with no WPC addition; while these values decreased when the addition level was increased to 4% (El-Magoli *et. al*., 1996). An extruded mix of WPC (80% protein) and cornstarch (2:1) was incorporated in a burger patty formulation. Patties containing 40% of extruded WPC showed less cooking loss, less cooking reduction and the same acceptance than all beef patties (Hale *et. al*., 2002).

Water holding capacity of poultry meat batters with no salt was increased when 4% preheated whey protein isolate (90.5%) was added to the formulation (Hongsprabhas and Barbut, 1999). Penetration force increased as WPI addition increased, compared with no addition of WPI. This indicates that preheated WPI helps in the binding of restructured poultry products (Hongsprabhas and Barbut, 1999). Whey protein isolates are widely used as a coating material. Emulsions of WPI and acetylated monoglycerides were used in spray coating of frozen king salmon by Stuchell and Krochta (1995). Less moisture loss and less peroxide values were found after 11 weeks of storage compared with no coating (Stuchell and Krochta, 1995).

Antimicrobial activity of whey protein isolate edible films was evaluated by Seydim and Sarikus (2006). Antimicrobial activity of spices were better expressed in WPI edible films than in products without affecting sensory properties (Seydim and Sarikus, 2006). Whey protein isolates are widely used by athletes due to the high protein content (> 90%) with a high bioavailable

amount of amino acids and fast absorption by the body (Chesley *et. al*., 1992, Kimball and Jefferson, 2006). Studies have found the potential of whey protein isolate to treat sarcopenia (muscle loss caused by aging) (Hayes and Cribb, 2008). Whey proteins provide essential amino acids, have the potential to act as a vitamin A precursor (de Wit, 1998) and have shown important advantages in the treatment and prevention of diseases (Ha and Zemel, 2003, Pal and Ellis, 2010, Pal *et. al*., 2010, Hamad *et. al*., 2011).

Whey protein powders are used for encapsulation of probiotic bacteria. Whey protein concentrate capsules (50% protein) were used by Rodrigues *et. al*. (2011) to encapsulate 3 probiotic strains (*Lactobacillus acidophilus Ki, Lactobacillus paracasei* L26 and *Bifidobacterium animalis* BB-12). In this study, 10% v/v of *Lactobacillus acidophilus Ki, Lactobacillus paracasei* L26 and *Bifidobacterium animalis* BB-12 were separately incorporated into a 50% WPC suspension or a 50% WPC + 0.5% L-cysteine suspension and microencapsulated (Rodrigues *et. al*., 2011). After encapsulation, microencapsulated and free cells of *Lactobacillus acidophilus Ki, Lactobacillus paracasei* L26 and *Bifidobacterium animalis* BB-12 were separately placed in perforated petri dishes and maintained in glass flasks at 5°C in the presence or absence of oxygen and different relative humidities (12, 32, 45%). Free cells of *Lactobacillus acidophilus Ki* did not survive after 60 days of storage at the conditions mentioned above but when encapsulation was applied using WPC, the survival was 10^7 cfu g^{-1} after 180 days at the storage conditions previously explained (Rodrigues *et. al*., 2011).

Other agents were used for encapsulation of probiotic bacteria such as pectin, cellulose and carrageenan (Gerez *et. al*., 2012). Whey protein was used by Gerez *et. al*., 2012 to coat

microencapsulated *Lactobacillus rhamnosus* CRL 1505. Uncoated *Lactobacillus rhamnosus* CRL 1505 were susceptible at low pH (1-2) at 60 minutes of incubation. In the other hand, coated viable *Lactobacillus rhamnosus* CRL 1505 were found at 120 minutes of incubation at low pH (1-2) (Gerez *et. al*., 2012). Up to 95% of survival cells were found when whey protein was used as encapsulation material (Gerez *et. al*., 2012).

1.1.2 Health Properties of Whey Proteins

Lipid accumulation in the liver (commonly known as fatty liver) consists in the infiltration of lipids into the hepatic cells in the liver (Schwimmer *et. al*., 2003, Chitapanarux *et. al*., 2009, Pal *et. al*., 2010, Hamad *et. al*., 2011, Petyaev *et. al*., 2012, Udenigwe and Aluko, 2012). This condition was previously linked with alcohol consumption but nowadays is commonly associated with overweight, diabetes, high carbohydrate diets, obesity, and insulin resistance and is known as Non-Alcoholic Fatty Liver Disease (NAFLD) (Chitapanarux *et. al*., 2009, Hamad *et. al*., 2011). Patients diagnosed with NAFLD are likely to develop pathogenesis such as heart failure, obesity, diabetes and metabolic risk factor syndrome of insulin resistance, high cholesterol, glucose intolerance, hypertension among others (Chitapanarux *et. al*., 2009, Pal *et. al*., 2010, Petyaev *et. al*., 2012).

Recent studies on animals and humans have found the effectiveness of supplementing whey protein products in the treatment and prevention of liver and metabolic diseases (Kent *et. al*., 2003, Madureira *et. al*., 2007, Chitapanarux *et. al*., 2009). NAFLD could lead to Non-Alcoholic Steatohepatitis (NASH) and subsequently to cirrhosis and hepatocellular carcinoma (Chitapanarux *et. al*., 2009). Reduction of Glutathione levels is associated with anti-oxidation

imbalance and liver diseases (Kent *et. al*., 2003, Chitapanarux *et. al*., 2009). Supplementation of rich-cysteine whey protein isolate to diets of NAFLD and NASH patients reduced hepatic steatosis in more than 60% of the patients (Chitapanarux *et. al*., 2009).

Whey protein isolate was used by Kent *et. al*. (2003) to reduce dead prostate cells. Prostate cells where protected from oxidant-induced cell death after the level of intracellular glutathione was increased when whey protein isolate was supplied during cell incubation (Kent *et. al*., 2003). β-LG is an important source of cysteine which stimulates the production of anticarcinogenic compounds, providing prevention of gastrointestinal track cancer and it also has beneficial effects in yogurt and probiotic bacteria (Dave and Shah, 1998a).

Glutathione (GSH) is a peptide found in cells of mammals and is responsible for the protection against oxidative agents (Kent *et. al*., 2003, Madureira *et. al*., 2007). When illness occurs, GSH is depleted because of cellular stress. Cysteine, glutamate and glycine are part of the primary structure of Glutathione (GSH). These amino acids are important in the T-cell response of macrophages and lymphocytes (Madureira *et. al*., 2007). Whey proteins are rich in cysteine and glutamate and the consumption of whey proteins or products containing whey proteins can increase the level of cysteine and help in the synthesis of GSH, which acts as a protective oxidative agent in immune system regulation and protection against formation of cancer cells (Madureira *et. al*., 2007). Besides cancer prevention, GSH is important for immune-enhancing effects of the immune system, liver functions and Alzheimer treatment (Madureira *et. al*., 2007). Besides the health and nutritional properties of whey proteins, these proteins could also provide more effects in food matrixes such as sensory, physical, chemical, and microbiological changes.

1.2 Yogurt

Yogurt is a fermented product made from homogenized and pasteurized milk inoculated with viable lactic cultures containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. According to The Code of Federal Regulations Section 131.206 the composition of fat free yogurt should be less than 0.5% of milk fat and 8.25% of MSNF (Milk Solids Non-Fat), and titratable acidity of lactic acid of 0.9% or greater (Lucas *et. al*., 2004). The metabolism and action of lactic acid bacteria produce volatile compounds, such as acetaldehyde, ethanol, acetone, diacetyl and 2-butanone, responsible for the flavor profile of the final product (Granata and Morr, 1996, Gardini *et. al*., 1999, Güler-Akin *et. al*., 2009). The interaction of these compounds provides characteristic flavors to the product making it acceptable or not to consumers (Güler-Akin *et. al*., 2009).

Milk products such as yogurt have an important market worldwide. Sensory characteristics such as flavor and aroma are improved by the addition of additives and compounds to dairy products, resulting in an increase in the acceptability of yogurt by consumers worldwide (Vinderola *et. al*., 2002). Despite the common use of natural and artificial additives added to foods in order to improve shelf life and some sensory characteristics, these additives can have effects on the viability of probiotics and starter culture bacteria present in the yogurt (Vinderola *et. al*., 2002). Additives such as whey proteins, casein, lactose, ethanol, inulin, starch and others can be added to yogurt (Dave and Shah, 1998a, Vinderola *et. al*., 2002, Mena and Aryana, 2012).

1.3 Lactic Acid Bacteria

According to Salminen *et. al*. (2004), lactic acid bacteria (LAB) are classified as gram-positive bacteria, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactic acid bacteria must be tolerant to stress existing while its path through the gastrointestinal track. After ingestion, these bacteria must overcome the low pH environment and presence of bile salts in the lower gastrointestinal track to have a beneficial effect in the host and be considered as a probiotic bacteria (Charteris *et. al*., 1998, Liong and Shah, 2005, Vernazza *et. al*., 2006).

Lactic acid bacteria are classified according to morphology, type of glucose fermentation and lactic acid produced, optimum growth temperature, tolerance to acid and alkaline environments and salt concentrations (Salminen *et. al*., 1999, Salminen *et. al*., 2004). Lactic acid bacteria are typically conformed by low-proteolytic activity bacteria, this bacteria uses carbohydrates as their main energy source, metabolizing them and producing lactic and acetic acids (Salminen *et. al*., 2004). *Streptococcus* and *Lactobacillus* genera are among the main lactic acid bacteria used in dairy foods (Nadal *et. al*., 2010).

1.3.1 Probiotics

Probiotics are live bacteria added to food products that provide health benefits (FAO, 2001). In order to impart these desired benefits, these bacteria have to survive severe conditions of pH and bile in the gastrointestinal track (Gerez *et. al*., 2012). Functional foods including probiotic bacteria are gaining popularity due to the health benefits related with probiotic consumption and the concept of preventive disease treatment (Leatherhead Foods International, 2011, Pedretti,

2013). The global sales of probiotics reached $21.6 billion and $24.23 billion in 2010 and 2011 respectively (Pedretti, 2013). The market of probiotic products is expected to reach $31.1 billion and $44.9 billion in 2015 and 2018 respectively (Pedretti, 2013). The actual health and economic importance of probiotic products and the expected market growth creates an important field to study the behavior of probiotics in dairy products. Some health effects provided by the consumption of lactic acid bacteria are: (1) improvement of gastrointestinal tract health, (2) improvement of lactose metabolism and reduction of lactose intolerance symptoms, (3) enhancement of immune system, (4) treatment of bacterial infection in gastrointestinal track (Shah, 2007).

Some requirements have to be met by lactic acid bacteria in order to be considered probiotics. The lactic acid bacteria should be a normal inhabitant of the human gastrointestinal tract, survive the passage through the upper digestive track in large numbers and have a beneficial effect in the gastrointestinal track (Turgut and Cakmakci, 2009, Nadal *et. al*., 2010). Products claiming to contain probiotic bacteria should have a concentration of 10^6-10^7 CFU per gram of viable probiotic bacteria in the final product (FAO/WHO, 2001).

1.3.2 Health Benefits of Culture and Probiotic Bacteria

It is well known that yogurt and fermented milk products are rich in beneficial bacteria. Lactic Acid Bacteria (LAB) are added during the manufacture of the product in order to start the fermentation process. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* are commonly used as starter cultures in yogurt and are considered as potential probiotic bacteria (Nadal *et. al*., 2010).

Anticarcinogenic effect and lactose tolerance improvement are other beneficial health properties associated with probiotic and culture bacteria consumption (Guarner and Malagelada, 2003, Shah, 2007). Yogurt culture bacteria (*L. bulgaricus and S. thermophilus*) used for yogurt manufacture also provided health benefits by giving a protective effect against DNA damage in organs caused by heterocyclic amines (Zsivkovits *et. al*., 2003). Animals assays showed that pre-carcinogenic induced lesions in colon cells of rats were reduced when diets where supplemented with a suspension containing *Lactobacillus bulgaricus* 291 (Zsivkovits *et. al*., 2003).

Consumption of formula containing *S. thermophilus* was used to treat gastrointestinal problems in healthy infants (4-9 months old) (Thibault *et. al*., 2004). Infant formula was fermented by Thibault *et. al*. (2004) using *Streptococcus thermophilus 065* and *Bifidobacterium breve C50.* Infants fed with fermented formula showed less diarrhea episodes, less hospitalization, fewer prescriptions and less dehydration compared with those fed with standard formula without the presence of *Streptococcus thermophilus 065* and *Bifidobacterium breve C50* (Thibault *et. al*., 2004).

1.4 Effect of Whey Proteins on Probiotic and Culture Bacteria

Recent studies have proved the potential of whey proteins to enhance the survival and viability of probiotic and culture bacteria (Akalin *et. al*., 2007, Ummadi and Curic-Bawden, 2008, Doherty *et. al*., 2010, 2011, Rodrigues *et. al*., 2011, Doherty *et. al*., 2012). Probiotics and culture bacteria are linked with health benefits once they reach and colonize the lower gastrointestinal track. In order to impart these desired benefits, these bacteria have to survive severe conditions of pH and bile in the gastrointestinal track (Gerez *et. al*., 2012). Other requirements are oxygen

and heat tolerance, ability to grow in milk, and metabolize prebiotics. Sensory characteristics of the final product do not have to be adversely affected (Nadal *et. al*., 2010), (Turgut and Cakmakci, 2009).

The effect of addition of milk derivatives (cysteine, whey powder, casein, whey protein concentrate, and whey protein isolate) on the growth and survival of probiotic and culture bacteria was evaluated by Charteris *et. al*., (1998), Akalin *et. al*., (2007), Almeida *et. al*., (2009), and Marafon *et. al*., (2011). Viability of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Bifidobacterium animalis* in reduced-fat yogurt supplemented with 1.5% of whey protein concentrate (WPC) was increased up to 1 log cfu/g after 1 week of storage compared with no supplementation with WPC (Akalin *et. al*., 2007). According to Akalin *et. al*. (2007) the buffer capacity of whey protein concentrate slowed down the product acidification during shelf life, thus protecting the probiotic and yogurt culture bacteria from high acid environments. According to Nadal *et. al*. (2010) in order to increase the total count of probiotic bacteria in the final product, the pH has to be >4.6, which could be maintained by the addition of whey protein powders.

When specific amino acids such as cysteine are added to yogurt, a significantly increase in viability of culture and probiotic bacteria (*Lactobacillus bulgaricus ssp. delbrueckii, L. acidophilus, Bifidobacterium bifidum* BB12 and *Lactobacillus paracasei*) was found (Güler-Akin and Akin, 2007). Yogurt culture bacteria *Streptococcus thermophilus* was affected by the addition of pure cysteine to the yogurt mix (Güler-Akin and Akin, 2007).

Addition of 0.5% whey protein concentrate (WPC) and other milk ingredients to replace non-fat dry milk resulted in an increase *of Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Bifidobacterium animalis* counts after 14 days of storage at 4°C (Marafon *et. al*., 2011). In the same study, after 28 days of storage at 4°C, counts of *Streptococcus thermophilus* and *Bifidobacterium animalis* were higher in yogurt supplemented with 0.5% WPC than counts in yogurt with added skim milk (Marafon *et. al*., 2011). Cell counts *of L. rhamnosus, L. bulgaricus, Bifidobacterium lactis* and *S. thermophilus* fermented in mixes of whey and milk containing 8% and 10% of total solids showed higher growth compared with growth when fermented in a mix (12% total solids) of milk with no added whey (Almeida *et. al*., 2009).

Addition of whey protein concentrate, whey protein isolate and caseinate in yogurt formulations are done to increase the total solid content and to improve rheological and sensory characteristics (Isleten and Karagul-Yuceer, 2006, Patocka *et. al*., 2006, Kücükcetin, 2008). These formulation changes can affect the behavior of cultures and probiotic bacteria as explained by Dave and Shah (1998b). The influence of different whey derivatives on the viability of *Lactobacillus acidophilus, Streptococcus thermophilus* and Bifidobacteria was evaluated during storage of yogurt. This yogurt was supplemented with L-cysteine (0, 50, 250, and 500 mg/L), 2% whey powder, 2% whey protein concentrates (WPC), 250 mg/L casein hydrolysate or 250 mg/L tryptone. After 5 weeks of storage at 4°C, counts of *Streptococcus thermophilus* in yogurt supplemented with whey protein concentrates and casein hydrolysate were up to 0.5 log CFU/mL higher compared with no supplementation (Dave and Shah, 1998a). Bifidobacteria counts were 4 log CFU/mL higher in yogurt supplemented with WPC compared to counts in

yogurt with no supplementation of WPC. Bifidobacteria remained at therapeutic levels when yogurt was supplemented with 2% WPC (Dave and Shah, 1998a).

The protection offered by the interaction between whey derivatives and probiotic bacteria is strain dependent (Dave and Shah, 1998a). Fortification of yogurt with up to 4% of whey protein hydrolysates improves the growth of *Lactobacillus acidophilus* by 3 log and enhances the growth of *Streptococcus thermophilus*, while it decreases the growth of *Lactobacillus rhamnosus* (Lucas *et. al*., 2004). Supplementation of yogurt with cysteine (up to 500 mg/L) improved the viability of *L. acidophilus* by 1.3 log CFU/mL but it affected the viability of *S. thermophilus* after 35 days of storage at 4°C (Dave and Shah, 1998a).

Whey protein isolate (0.1%) was used to improve survival of potentially probiotic strains of *Lactobacillus casei* 212.3 and *Bifidobacterium infantis* 25962 in an in vitro assay to evaluate survival after a simulated gastric transit tolerance test (Charteris *et. al*., 1998). Cells of *Lactobacillus rhamnosus GG* were entrapped using native, heat treated and hydrolyzed whey protein isolate and added to a commercial low-fat plain yogurt (10^8 CFU/mL) and stored at 10°C during 14 days (Doherty *et. al*., 2010). The use of native and heat treated WPI to entrap cells of *Lactobacillus rhamnosus GG* showed an increase of ≈0.5 log CFU/g in the bacterium strain when added to yogurt compared with viability of free cells added to yogurt (Doherty *et. al*., 2010). The influence of growth and survival of starter culture bacteria in an enriched WPI yogurt matrix is not well understood.

Although differences were found in survival comparing different strains of probiotics, overall the use of whey protein in yogurt and for encapsulation of probiotic bacteria resulted as an effective agent to prevent mortality of viable cells (Dave and Shah, 1998b, Dave and Shah, 1998a, Almeida *et. al*., 2009, Doherty *et. al*., 2010, Weinbreck *et. al*., 2010, Doherty *et. al*., 2011, Rodrigues *et. al*., 2011, Doherty *et. al*., 2012, Gerez *et. al*., 2012).

1.5 Whey Protein Concentrate in Yogurt Manufacture

The effect of whey protein concentrate (35% protein), microparticulated whey protein, anhydrous milk fat and tapioca starch on the physical characteristics of yogurt was evaluated by Sandoval-Castilla *et. al*. (2004). Yogurt with 1% WPC added resulted in similar physical characteristics as yogurt made with standardized milk (1.5% fat). Less firmness was observed when 1% starch was used instead of WPC (Sandoval-Castilla *et. al*., 2004). According to Sandoval-Castilla *et. al*., (2004), a linked protein structure was observed in yogurt supplemented with 1% WPC compared with a spacious and loose structure when yogurt was not supplemented with WPC (Sandoval-Castilla *et. al*., 2004). Martín-Diana *et. al*. (2003) found improvement in sensory characteristics of set type yogurt when whey protein concentrate (35% protein) was added. Syneresis reduction, higher apparent viscosity and gel firmness were found when WPC was added (Martín-Diana *et. al*., 2003).

1.6 Whey Protein Isolate in Yogurt Manufacture

Whey protein isolates are used in manufacture of dairy products as a gelling agent in yogurt and emulsifying agent in ice cream, also to increase protein content of yogurt and ice cream mixes and to improve rheological characteristics of yogurt (de Wit, 1998, ADPI, 2002, Puvanenthiran *et. al*., 2002, Patocka *et. al*., 2006, Kücükcetin, 2008). Low gelation level, low acidification, and

lack of yogurt flavor were found in a soy-based yogurt. The acidification level was improved when 3.5% WPI and caseinate were added to the soy-based yogurt mix (Karleskind *et. al*., 1991). Inclusion of dairy derivatives (casein, WPI, caseinate and NFDM) provide important nutrients for the growth of starter culture bacteria, therefore, yogurt physical and sensorial characteristics can be improved (Karleskind *et. al*., 1991).

The effect of heat and casein-to-whey protein isolate (93.5% protein) ratio was evaluated by Kücükcetin (2008), who prepared stirred yogurt with a yogurt mix containing 1.5:1 to 4:1 casein to whey protein isolate ratio. When the casein to WPI ratio was lower, higher visual roughness, number of grains and yield stress were found compared to a higher casein to WPI ratio. Less syneresis was found when the casein to whey protein ratio was 1.5:1 and 2:1 (Kücükcetin, 2008).

Patocka *et. al*. (2006) studied WPI (90% protein) addition before and after fermentation of a stirred yogurt mix. In the same study, commercial drink yogurt and commercial stirred yogurt were obtained from local stores and WPI (90% protein) was added to evaluate their rheological behavior. Addition of up to 10% WPI to prior manufactured commercial drink yogurt resulted in an 80% decrease of apparent viscosity compared with commercial drink yogurt without WPI supplementation (Patocka *et. al*., 2006). Addition of WPI (1-3%) to prior manufactured commercial stirred yogurt did not affect the structure and viscosity compared with commercial stirred yogurt without WPI supplementation (Patocka *et. al*., 2006). In addition, plain yogurt was prepared and WPI was added after pasteurization of yogurt mix but before or after fermentation (Patocka *et. al*., 2006). Addition of WPI above 6% after pasteurization but before fermentation resulted in a reduction of apparent viscosity of plain yogurt compared with plain yogurt without

WPI supplementation (Patocka *et. al*., 2006). In contrast, addition of WPI (<6%) after pasteurization to a plain yogurt mix but before fermentation showed similar apparent viscosity compared to plain yogurt without WPI supplementation (Patocka *et. al*., 2006). When WPI (>4%) was added to a yogurt mix after pasteurization and after fermentation resulted in a separation of phases and aggregation of solids (Patocka *et. al*., 2006). Although different WPI supplementation levels and different conditions for addition were evaluated, the study was focused only on the rheological behavior of WPI addition to different food systems. They did not evaluate the effect on microbiological properties and other yogurt properties.

The effect of addition of dry ingredients to a yogurt mix was evaluated by Isleten and Karagul-Yuceer (2006). In this study 1% WPI (93% protein content) was added to a yogurt mix. Other treatments included addition of 1% of other dry ingredients (Skim milk powder and sodium caseinate). Physical and sensory characteristics were evaluated. Higher apparent viscosity values were reported by Isleten and Karagul-Yuceer (2006) when 1% WPI was added to the yogurt mix compared with no addition of WPI. Addition of 1% WPI reduced syneresis up to 50% compared with no supplementation of WPI (Isleten and Karagul-Yuceer, 2006). Lumpiness (visual perception of grains) and Chalkiness (particle perceptions in mouth) were higher in yogurt with 1% WPI added compared with yogurt supplemented with skim milk and sodium caseinate (Isleten and Karagul-Yuceer, 2006). While sensory and physical characteristics were evaluated by Isleten and Karagul-Yuceer (2006), the effect of different amounts of WPI supplementation on other important yogurt characteristics such as pH and titratable acidity were not studied and microbiological properties of yogurt culture bacteria were also not evaluated.

Physical properties of goat's milk yogurt were evaluated when polymerized and native whey protein isolate (93% protein) was used in the yogurt mix before pasteurization (Li and Guo, 2006). A dispersion containing 2.4% of WPI was prepared and a portion was preheated at 90°C for 30 minutes (polymerized dispertion). Dispersions were added separately in yogurt mix. After yogurt manufacture, syneresis was reduced by 25% when yogurt was supplemented with preheated WPI dispersion compared with no supplementation and native WPI supplementation (Li and Guo, 2006). Higher viscosity was found in yogurt with added polymerized (preheated) whey protein isolate (Li and Guo, 2006) but the microbiological properties of yogurt culture bacteria were not evaluated. Yogurt characteristics (pH, titratable acidity) over storage were also not evaluated.

1.7 Justification

The global whey trade was 1,257,054 metric tons (MT) in 2011 (Stiles, 2012). The global production of whey milk powders was 4.2 million MT in 2011 (Lafougère, 2012). The global market of whey proteins was USD 3.8 billion in 2008 and USD 5 billion in 2010 with an expected growth to USD 6.14 billion in 2014 (Leatherhead Foods International, 2011).

Functional foods are those that have health benefits above and beyond traditional foods. The global sales of functional foods reached $24.2 billion USD in 2010, and increase of 5% compared with sales in 2009 (Leatherhead Foods International, 2011). The market of functional foods is expected to increase up to $130 billion in 2015, including $29 billion in probiotic and probiotic products sales by 2015 (Leatherhead Foods International, 2011).

Whey protein concentrates and whey protein isolates are used for muscle recovery after weight lifting and work out routines of athletes and body builders (Tipton *et. al*., 2007). WPC and WPI are sources of branched chain amino acids (BCAA) leucine, isoleucine and valine, three essential amino acids (Sowers, 2009). BCAA enter to the bloodstream through the liver and are oxidized in muscle tissue to provide energy (Garlick and Grant, 1988, Morifuji *et. al*., 2009, Sowers, 2009). Muscle tissue use BCAA amino acids as energy source during exercise (Manninen, 2009, Morifuji *et. al*., 2009, Sowers, 2009). Products containing BCAA can reduce muscle degradation and improve workout performance (Manninen, 2009, Sowers, 2009). The timing and source of whey protein concentrates and isolates are factors affecting the anabolic response of muscle recovery (Tipton *et. al*., 2007). There is an increasing importance of diets containing less carbohydrates and fats and more protein (Weigle *et. al*., 2005, Wycherley *et. al*., 2010). Prevention of cancer and diabetes, weight loss and reduction of appetite are linked with a low carbohydrate and high protein diets (Weigle *et. al*., 2005, Wycherley *et. al*., 2010).

The effect of WPI on probiotic characteristics of pure bacterial cultures and culture bacteria in a yogurt matrix are not known. Also, the effects of incremental addition of WPI on bacterial and yogurt characteristics are not well understood.

The hypothesis was that "added" whey protein isolate will influence the characteristics of yogurt culture bacteria in pure form and in yogurt. The objectives of this study were:

1. To study the effect of whey protein isolate on acid tolerance, bile tolerance, growth and protease activity of pure cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus.*

2. To study the effect of whey protein isolate on acid tolerance, bile tolerance and growth of yogurt cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* from fat free plain yogurt.

3. To evaluate the effect of incremental addition of whey protein isolate on the properties (apparent viscosity, pH, titratable acidity, syneresis) of a fat free plain yogurt weekly over 35 days of storage at 4°C.

4. To evaluate the effect of incremental addition of whey protein isolate on sensory characteristics of blueberry flavored yogurt.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Design

The treatments consisted of three concentrations of added whey protein isolate (1, 2 and 3% w/v). The control did not have whey protein isolate added (0%).

Acid tolerance on pure cultures (*Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12) was evaluated every 30 minutes for 2 hours and bile tolerance on pure culture was determined every hour for 5 hours. Growth was determined on pure culture every 12 hours for 60 hours. Protease activity of pure cultures was separately evaluated at 0, 12 and 24 hours of incubation. The above experiments were conducted and analyzed as Repeated Measure Design. Three replications were conducted.

Fat free plain yogurts were manufactured with 0, 1, 2 and 3% WPI. Acid tolerance was evaluated on 7 day old fat free plain yogurt every 30 minutes for 2 hours while bile tolerance was determined every hour for 5 hours. Growth of starter bacteria in fat free plain yogurt was determined at 7, 21 and 35 days of storage at 4°C. The above experiments were conducted and analyzed as Randomized Block Design. Three replications were conducted. Repetitions were the blocks.

Fat free plain yogurt with added whey protein isolate (0, 1, 2 and 3% w/v) was evaluated for apparent viscosity, pH, titratable acidity and syneresis at days 1, 7, 14, 21, 28 and 35 of storage. Three replications (blocks) were evaluated in a Randomized Block Design (RBD).

A sensory study for consumer acceptance of blueberry yogurt with added whey protein isolate was performed with 100 panelists; panelists were the blocks. This study was conducted and analyzed as a Randomized Block Design (RBD).

2.2 Yogurt Manufacture

This study was focused on incremental additions of whey protein isolate. Two types of yogurt were manufactured. Added whey protein isolate fat free plain yogurt was manufactured and used for physico-chemical and microbiological analyses. No flavorings were added to eliminate interference of flavoring components on analyses. The second yogurt manufactured had whey protein isolate with blueberry yogurt for sensory evaluation of the final overall product.

Fat free plain yogurt was manufactured according to standard procedure at the Louisiana State University Dairy Processing Plant. Whey protein isolate powder (Grände Custom Ingredients Group, Milwaukee, WI, USA) was added at 0 (control), 1, 2, and 3% w/v to the yogurt mixture contained in previously cleaned and sanitized pails. Control and added whey protein isolate yogurt mixtures had 3% w/v Non-fat Dry Milk (NFDM) added to the mixture.

The milk was preheated at 50°C and the solids were added. The mix was heated at 60°C then homogenized in a two stage homogenizer (Type: 300 DJP4 2PS, Gaulin, Manton-Gaulin MFG Co Inc., Everett, MA, USA) at 13.8 MPa for the first stage and 3.45 MPa for the second stage and later pasteurized at 65ºC for 30 minutes (Savaiano *et. al*., 1984). Yogurt mix formulations are reported in Table 1.

Yogurt mixtures were cooled to 40°C and inoculated. Freshly thawed frozen yogurt starter culture concentrate of *Streptococcus thermophilus* (ST-M5) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB-12) (Chr. Hansen´s Laboratory, Milwaukee, WI, USA) was added at 0.75 mL per 3.78 L of milk for each bacterial strain, 11.34 L of skim milk per treatment were used. After mixing, the yogurt mix was poured into previously labeled plastic cups. The inoculated mixture was incubated at 40 °C until pH reached 4.65 ± 0.1 to obtain a fat free plain yogurt, and transferred to the cooler at 4ºC for refrigeration until further analyses. Yogurt manufacture was replicated 3 times.

Table 1. Fat free plain set-type yogurt formulations

INGREDIENTS	WHEY PROTEIN ISOLATE (WPI) CONCENTRATIONS			
	0%	1%	2%	3%
Skim Milk	11.34 L	11.34 L	11.34 L	11.34 L
Non-fat Dry Milk	340.2 g	340.2 g	340.2 g	340.2 g
WPI	0 g	113.4 g	226.8 g	340.2 g
Starter Culture	4.5 mL	4.5 mL	4.5 mL	4.5 mL

A separate batch of blueberry flavored yogurt with the same control (0% w/v WPI) and added whey protein isolate treatments (1, 2, 3% w/v WPI) was manufactured for sensory evaluation. The manufacture process used was the same with the exception that 15% w/w blueberry puree was added after plain yogurt manufacture and refrigerated at 4ºC.

2.3 Preparation of Media

2.3.1 Peptone water

Peptone water (0.1%) was prepared according to manufacturer specifications by dissolving 1g of peptone powder (BactoTM Peptone, Difco, Dickinson and company, Sparks, MD) in 1L of distilled water. Peptone water was autoclaved in 99mL bottles at 121°C for 15 minutes.

2.3.2 Acid tolerance broths

MRS broth for acid tolerance was prepared by adding 55g of MRS broth powder (DifcoTM, Dickinson and company, Sparks, MD) to distilled water (1 L). M17 broth for acid tolerance was prepared by adding 37.25g of M17 (Oxoid, Basingstoke, UK) to 950 mL of distilled water. A solution of 1N HCL was used to adjust broths at pH 2. A calibrated pH meter (Extech Instruments, Waltham, MA) was used to adjust MRS and M17 broths to pH 2 using 1 N HCL. The MRS and M17 broths were sterilized at 121°C for 15 sec. A lactose solution (10% w/v) was sterilized and aseptically added to previously sterilized M17 broth.

2.3.3 Bile tolerance broths

MRS-THIO broth for bile tolerance was prepared by adding 55g of MRS broth powder (DifcoTM, Dickinson and company, Sparks, MD), bovine bile (0.3% (w/v) oxgall) (US Biological, Swampscott, MA) and 0.2 % (w/v) sodium thioglycolate (Acros Organics, Fair Lawn, NJ)] to distilled water (1 L). Sodium thioglycolate was used as oxygen scavenger for *L. bulgaricus* in MRS broth. The final pH of the broth was 6.70±0.2. M17 broth for bile tolerance was prepared by adding 37.25g of M17 (Oxoid, Basingstoke, UK) to 950 mL of distilled water and supplemented with bovine bile (0.3% (w/w) oxgall) (US Biological, Swampscott, MA). The

final pH of the broth was 7.00±0.2. The MRS-THIO and M17 broths were sterilized at 121°C for 15 minutes. A lactose solution (10% w/v) was sterilized and aseptically added to previously sterilized M17 broth.

2.3.4 Broths for enumeration of culture bacteria

MRS broth for enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus* was prepared by adding 55g of MRS broth powder (DifcoTM, Dickinson and company, Sparks, MD) to distilled water (1 L). The MRS broth was sterilized at 121°C for 15 minutes. M17 broth for enumeration of *Streptococcus thermophilus* was prepared by adding 37.25g of M17 (Oxoid, Basingstoke, UK) to 950 mL of distilled water. The MRS and M17 broths were sterilized at 121°C for 15 minutes. A lactose solution (10% w/v) was sterilized and aseptically added to previously sterilized M17 broth.

Whey protein isolate powder (Grände Custom Ingredients Group, Milwaukee, WI, USA) was separately added at 0 (control), 1, 2, and 3% (w/v) to all broths described above.

2.3.5 *Lactobacilli* MRS agar

MRS agar was prepared according to manufacturer's directions. MRS broth powder (DifcoTM, Dickinson and company, Sparks, MD) (55g) and agar (15g) were added to distilled water (1 L) and pH was adjusted to 5.2. The MRS agar was sterilized at 121°C for 15 minutes.

2.3.6 M17 agar

M17 agar was prepared according manufacturer's directions by adding 37.25g of M17 (Oxoid,

Basingstoke, UK) and agar (11g) to 950 mL of distilled water. M17 agar was sterilized at 121°C for 15 minutes. A lactose solution (10% w/v) was sterilized and aseptically added to previously sterilized M17 agar.

2.3.7 *Streptococcus thermophilus* agar

Streptococcus thermophilus agar was prepared by the addition of tryptone (10 g), sucrose (10 g), yeast extract (5 g) and potassium phosphate (2g) to distilled water (1 L). A calibrated pH meter (Extech Instruments, Waltham, MA) was used to adjust pH to 6.8±0.1 using 1 N HCL. Then, bromocresol purple 0.5% (6 mL) was added. The *Streptococcus thermophilus* agar was sterilized at 121°C for 15 minutes.

2.4 Microbiological Analysis

2.4.1 Acid tolerance procedure for pure culture bacteria

Acid tolerance was determined according to the method proposed by Pereira and Gibson (2002), with slight modifications. Freshly thawed pure cultures of S. *thermophilus* ST-M5 and *L. bulgaricus* LB-12 (1% v/v) were used to separately inoculate M17 broth pH 2 (Oxoid, Basingstoke, UK) and MRS broth pH 2 (DifcoTM, Dickinson and company, Sparks, MD), respectively. These broths contained 0 (control), 1, 2 and 3% w/v added WPI. Inoculated broths (pH 2) containing control and added WPI samples were incubated during 2 hours at 43°C for *L. bulgaricus* and 37°C for *S. thermophilus*.

Inoculated MRS (pH 2) and M17 (pH 2) broths were tenfold serially diluted in peptone water (0.1% w/w) and pour plated in duplicate every 30 minutes during the 2 hours of incubation.

Lactobacillus bulgaricus was enumerated by pour plating using previously prepared MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using previously prepared M17 agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted.

2.4.2 Acid tolerance procedure for culture bacteria in fat free plain yogurt

Acid tolerance of culture bacteria in fat free plain yogurts containing 0, 1, 2, and 3% WPI was analyzed at day 7 of storage. Fat free plain yogurt (1g) was added to an acidified broth (pH 2) containing previously prepared MRS pH 2 (DifcoTM, Dickinson and company, Sparks, MD) and M17 pH 2 (Oxoid, Basingstoke, UK) broths in a 1:1 ratio. The broth was incubated at 40°C for 2 hours and pours plated every 30 minutes during the incubation time.

Inoculated broths (MRS pH 2 and M17 ph 2 in a 1:1 ratio) were tenfold serial diluted in peptone water (0.1% w/w) and plated in duplicate every 30 minutes during the 2 hours of incubation. *Lactobacillus bulgaricus* was enumerated by pour plating using MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using *Streptococcus thermophilus* agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted. Three replications were performed.

2.4.3 Bile tolerance procedure for pure culture bacteria

Bile tolerance was determined according to the method proposed by Pereira and Gibson (2002)

and Dave and Shah (1996), with slight modifications. Freshly thawed pure cultures of S. *thermophilus* ST-M5 and *L. bulgaricus* LB-12 (10% v/v) were used to separately inoculate MRS-THIO broth (0.3% w/v oxgall) for *L. bulgaricus* and M17 broth (0.3% w/v oxgall) for *S. thermophilus.* Oxgall was added to test bile tolerance of both bacteria and 0.2 % (w/v) sodium thioglycolate (Acros Organics, Fair Lawn, NJ) was added as oxygen scavenger to the MRS-THIO broth for *Lactobacilli* only. These broths contained 0 (control), 1, 2 and 3% w/v added WPI. Inoculated broths containing control and WPI samples were incubated during 5 hours at 43°C for *L. bulgaricus* and 37°C for *S. thermophilus*.

Inoculated MRS-THIO (0.3% w/v oxgall) and M17 (0.3% w/v oxgall) broths were tenfold diluted in peptone water (0.1% w/w) and pour plated every hour during the 5 hours of incubation. *Lactobacillus bulgaricus* was enumerated by pour plating using previously prepared MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using previously prepared M17 agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted. Three replications were performed.

2.4.4 Bile tolerance procedure for culture bacteria in fat free plain yogurt

Bile tolerance of culture bacteria in fat free plain yogurts containing 0, 1, 2, and 3% WPI was analyzed at day 7 of storage. Fat free plain yogurt (1g) was added to broth containing previously prepared MRS (0.3% w/v oxgall) and M17 (0.3% w/v oxgall) broths in a 1:1 ratio. The broth was incubated at 40°C for 5 hours and pours plated every hour during the incubation time. Inoculated broths (MRS [0.3% w/v oxgall] and M17 [0.3% w/v oxgall] in a 1:1 ratio) were tenfold serial

diluted in peptone water (0.1% w/v) and plated in duplicate every hour during the 5 hours of incubation. *Lactobacillus bulgaricus* was enumerated by pour plating using MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using *Streptococcus thermophilus* agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted. Three replications were performed.

2.4.5 Enumeration of pure culture bacteria

Growth was determined according to the method proposed by Dave and Shah, 1998a, with slight modifications. Freshly thawed pure cultures of *L. bulgaricus* LB-12 and *S. thermophilus* ST-M5 (1% v/v) were used to separately inoculate MRS and M17 broths, respectively. These broths contained 0 (control), 1, 2 and 3% w/v added WPI. Inoculated broths containing control and added WPI samples were incubated during 60 hours at 43°C for *L. bulgaricus* and 37°C for *S. thermophilus*. Inoculated MRS and M17 broths were tenfold serially diluted in peptone water (0.1% w/w) and pour plated in duplicate every 12 hours during the 60 hours of incubation. *Lactobacillus bulgaricus* was enumerated by pour plating using previously prepared MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using previously prepared *Streptococcus thermophilus* agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted.

2.4.6 Enumeration of culture bacteria in fat free plain yogurt

Enumeration of culture bacteria in fat free plain yogurts containing 0, 1, 2, and 3% w/v added

WPI was analyzed at day 7, 21 and 35 of storage at 4°C. Fat free plain yogurts (0, 1, 2, and 3% w/v WPI) (1g) were tenfold serially diluted in peptone water (0.1% w/w) and pour plated in duplicate. *Lactobacillus bulgaricus* was enumerated by pour plating using MRS agar (pH 5.2) (Dave and Shah, 1998a) and anaerobically incubated at 43°C for 72 hours. *Streptococcus thermophilus* was enumerated by pour plating using *Streptococcus thermophilus* agar and aerobically incubated at 37°C for 24 hours. After the incubation period, the colonies were counted. Three replications were performed.

2.4.7 Coliform Counts

The blueberry yogurt was tested for coliforms before conducting the sensory evaluation using petrifilms (3M®, St. Paul, MN) which contain violet red bile agar (VRBA). Blueberry yogurts containing 0 (control), 1, 2, and 3% w/v WPI were separately analyzed by weighting 11g of yogurt samples and pouring into a sterile bottle containing 99mL of sterile 0.1% peptone water (Difco, Detroit, MI, USA). Contents in bottle were agitated to prepare serial dilutions and 1mL was taken from dilutions 10^{-1} and 10^{-2} and plated in duplicate for control and added WPI samples. Previously labeled petrifilms were kept aerobically at 32°C for 24 hours.

2.4.8 Protease activity

The extracellular protease activity of pure cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 was determined using the *o*-phthaldialdehyde (OPA) spectrophotometric method proposed by Oberg *et. al*., (1991) with slight modifications. Pure cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 were inoculated (1% v/v) into sterile skim milk previously autoclaved at

121 ºC for 15 min, and incubated at 40 ºC for 0, 12 and 24 hours. Whey protein isolate powder (Grände Custom Ingredients Group, Milwaukee, WI, USA) was separately added at 0 (control), 1, 2, and 3% (w/v) to sterile skim milk. After incubation, 2.5 mL from each sample was mixed with 1 mL of distilled water and transferred into test tubes containing 5 mL of 0.75N trichloroacetic acid (TCA) (Fisher Scientific) and the content was mixed using a vortex. After sitting at room temperature for 10 minutes, the acidified samples were filtered through a Whatman Number 2 filter paper (Clifton, NJ). Duplicate aliquots from each TCA filtrate were analyzed by OPA testing using a spectrophotometer. The OPA final solution was prepared by combining the following reagents and diluting to a final volume of 50 mL with distilled water: 25 mL of 100 mM sodium borate (Fisher Scientific); 2.5 mL 20% (w/w) SDS (Fisher Scientific); 40 mg of OPA (Alfa Aesar, Ward Hill, MA) dissolved in 1 mL methanol (Sigma); and 100 µL of β-mercaptoethanol (Sigma). One hundred and fifty µL of each TCA filtrate was mixed with 3 mL of OPA reagent in a 3 mL cuvette, and the absorbance at 340 nm was read. Absorbance of the OPA final solution with the non-inoculated sterile skim milk (reference) was subtracted from each sample reading.

2.5 Analytical Procedures

2.5.1 pH

The pH of the yogurt was analyzed at 1, 7, 14, 21, 28, and 35 days of storage using a Oysters Series pH meter (Extech Instruments, Waltham, MA) calibrated using commercial buffers (pH 4 and pH 7 Fisher Scientific).

2.5.1 Titratable acidity

Yogurt (9 g) was weighted to determine titratable acidity by adding 5 drops of phenolphthalein indicator and titrating with 0.1 N NaOH until a color change to rose pink was observed and persisted for 30 seconds.

2.5.2 Apparent viscosity

Apparent viscosity of yogurt was determined according to Isleten and Karagul-Yuceer (2006) with slight modifications. Yogurt gel was broken to incorporate free whey and placed in refrigeration at 4°C for 2 hours before tested to relieve stress caused when the gel was broken and agitated. A viscometer Brookfield model DV-II, Brookfield helipath stand (Brookfield Engineering Laboratories, Inc. Stoughton, MA, USA) and the Windgather® 32 software was used. A T bar A spindle at 10 rpm was used. Data was collected using the Wingather® 32 software (Brookfield Engineering Lab Inc.). A total of 100 data points were collected and the average for each sample was used for statistical analysis. Three replications of each sample at 10°C±2°C after 1, 7, 14, 21, 28, and 35 days of storage were conducted.

2.5.3 Syneresis

Syneresis was measured in accordance with Tamime *et. al*. (1996), (Amatayakul *et. al*., 2006), Isleten and Karagul-Yuceer (2006) with slight modifications. Spontaneous whey separation was measured in yogurt samples by weighting a 12 oz. cup containing 260 g of yogurt samples. Cups containing samples were analyzed at 8°C ±2°C. The cups containing samples were kept at an angle of 45° and loss of whey out of the gel was measured by the amount of whey drained from

the cup. The cup was weighted again and % of syneresis was calculated. Three replications of each sample at 1, 7, 14, 21, 28, and 35 days of storage were analyzed.

2.6 Sensory study

The sensory study was exempted from continued oversight by The LSU Institutional Review Board with the IRB exemption number HE 13-15a (Appendix A). Fat free blueberry yogurts containing the four treatments (0, 1, 2, and 3% w/v added WPI) was used for sensory evaluation. A consumer acceptance test was conducted using 100 random participants. Each participant received an informed consent in which the potential risks of the study were explained. Each participant received 4 samples in three digits-random number coded 3.25oz. plastic cups. Water and non-salted saltine crackers were provided to the participants to clean their palates in between each sample. Single use spoons were provided for each sample. Participants were instructed not to discuss their findings with other participants during the evaluation. A 9-point scale evaluation questionnaire (Appendix B), where 1 = dislike extremely, 5 = neither like nor dislike and 9 = like extremely, was used. Participants were asked to evaluate each yogurt sample for the following attributes: Appearance, Color, Aroma, Taste, Thickness, Graininess and Overall liking.

The evaluation questionnaire also contained a question for acceptability, intent of purchase if the product were commercially available and intent of purchase knowing the product contained higher protein amounts.

2.7 Statistical analysis

Data were analyzed using Proc Mixed of the SAS® 9.3 program. Differences of least square means were used to determine significant differences at $P < 0.05$ for main effects (WPI and

time), and two way interaction effects (WPI * time). Significant differences were determined at $\alpha = 0.05$. Significant differences ($P < 0.05$) among the main effects were analyzed using Tukey's adjustment. Data was analyzed using Proc Mixed model of Statistical Analysis System (SAS®). Data from yogurt consumer study was also analyzed using Statistical Analysis System (SAS®). For the yogurt consumer study, ANOVA was conducted to analyze the questions with the 9-point scale (Peryam and Pilgrim, 1957).

CHAPTER 3: RESULTS AND DISCUSSION

SECTION 1: Pure Culture Bacteria

3.1 Acid Tolerance

3.1.1 *Streptococcus thermophilus* ST-M5

Acid tolerance of *Streptococcus thermophilus* ST-M5 as influenced by the addition of whey protein isolate (WPI) over incubation during 120 minutes is shown in Figure 1. Treatment*minutes interaction effect, treatment effect and minutes effect were significant (P<0.05) (Table 2).

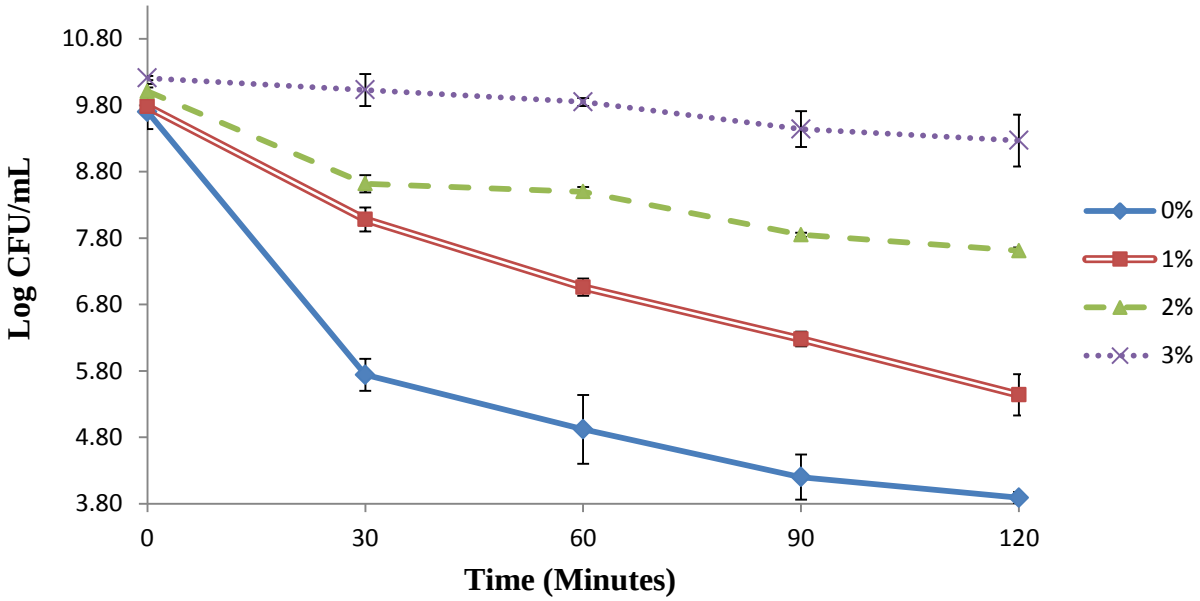

Figure 1. Acid tolerance of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 120 minutes.

Addition of 1, 2, and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to control from 30 to 120 minutes of incubation (Table 3). The 2 and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to control and 1% WPI at 60, 90, and

120 minutes of incubation (Table 3). The 3% WPI showed significantly (P<0.05) higher viable cell counts compared to 0, 1, and 2% WPI at 30, 60, 90, and 120 minutes of incubation (Table 3).

Mean log difference (Table 4) in the counts of *Streptococcus thermophilus* ST-M5 was obtained by subtracting log CFU/mL of 120 minutes from 0 minutes of incubation. A low number indicated lower bacterial death. Lower bacterial death was observed among samples containing 1, 2 and 3% WPI compared to control (Table 4). The bacterial death was the lowest for 3% WPI compared to the rest (Table 4). *Streptococcus thermophilus* ST-M5 showed a mean log difference of 5.81 CFU/mL in control (around 60% of bacterial death) compared to a mean log difference of 0.94 CFU/mL in 3% WPI (around 10% of bacterial death) (Table 4).

3.1.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

Acid tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by the addition of whey protein isolate (WPI) over incubation during 120 minutes is shown in Figure 2. Treatment*minutes interaction effect, treatment effect and minutes effect were significant (P<0.05) (Table 2).

Table 2. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate under the influence of acidic broth.

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	<0.0001
Minutes	<0.0001	<0.0001
Treatment*Minutes	<0.0001	<0.0001

Use of 1, 2, and 3% w/v WPI showed significantly (P<0.05) higher viable cell counts compared to control from 30 to 120 minutes of incubation (Table 3). The 2 and 3% WPI showed

significantly (P<0.05) higher viable cell counts compared to 0 and 1% WPI at 30, 60, 90, and 120 minutes of incubation (Table 3). Use of 3% WPI improved survival of *Streptococcus thermophilus* ST-M5 by 5 log CFU/mL and *Lactobacillus bulgaricus* LB-12 by 2 log CFU/mL after subjection to acidic conditions at 120 minutes (Table 3).

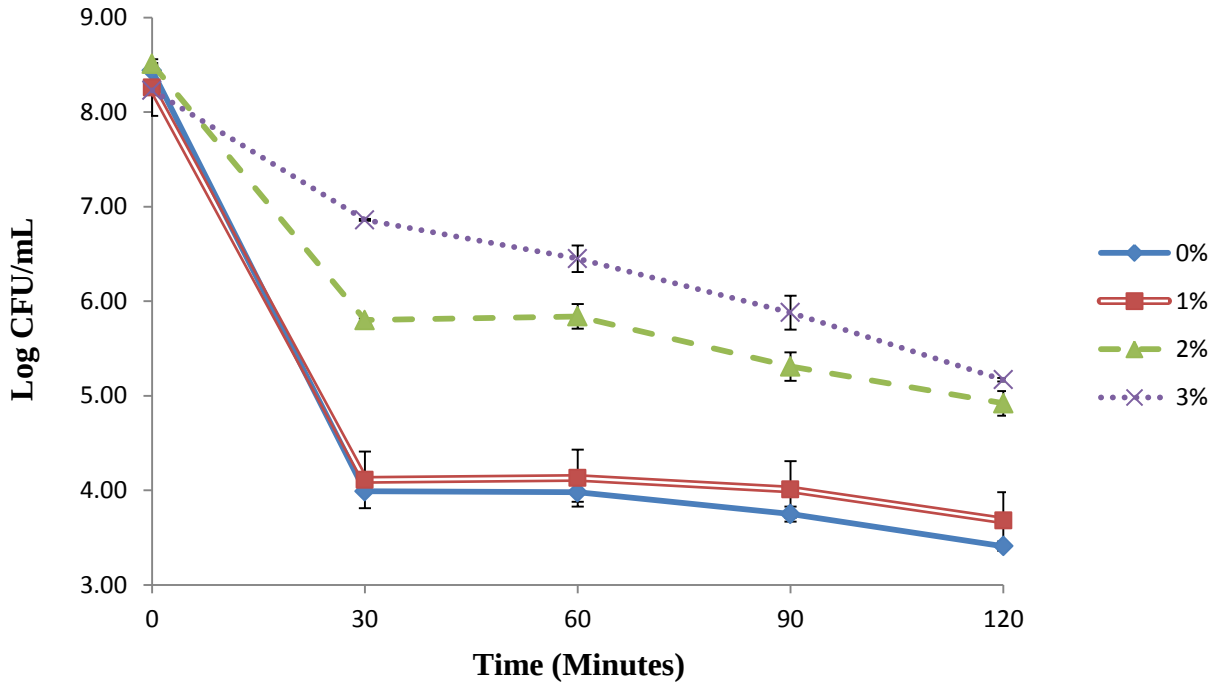

Figure 2. Acid tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 120 minutes.

Mean log difference (Table 4) in the counts of *Lactobacillus bulgaricus* LB-12 was obtained by subtracting log CFU/mL of 120 minutes from 0 minutes of incubation. A low number indicated lower bacterial death. Lower bacterial death was observed among samples containing 1, 2 and 3% WPI compared to control (Table 4). The bacterial death was the lowest for 3% WPI compared to the rest (Table 4). *Lactobacillus bulgaricus* LB-12 showed a mean log difference of 5.03 CFU/mL in control (around 60% of bacterial death) compared to a mean log difference of 3.06 CFU/mL in 3% WPI (around 37% of bacterial death) (Table 4).

Table 3. Least Square Means (Log CFU/mL) for acid tolerance of pure *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 120 minutes.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5					*Lactobacillus bulgaricus* LB-12				
	Time (Minutes)									
	0	30	60	90	120	0	30	60	90	120
Control	9.70 ^{ABC}	5.74 ^{IJ}	4.92 ^{K}	4.20 ^{L}	3.89 ^{L}	8.44^{A}	3.99^{J}	3.98^{J}	3.75^{K}	3.41^{L}
One	9.78 ^{ABC}	8.08 ^{EFG}	7.06 ^{H}	6.28 ^{I}	5.44 ^{JK}	8.26^{B}	4.11^{I}	4.13^{I}	4.01^{J}	3.68^{K}
Two	10.01^{AB}	8.62 ^{DE}	8.50 ^{EF}	7.85 ^{FG}	7.61 ^{GH}	8.51^{A}	5.80^{E}	5.84^{E}	5.31^{F}	4.92^{H}
Three	10.21^{A}	10.03^{AB}	9.85 ^{ABC}	9.44 ^{BC}	9.27 ^{CD}	8.23^{B}	6.86^{C}	6.45^{D}	5.88^{E}	5.17^{G}

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.

Table 4. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of acid.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
	Log CFU/mL	
Control	5.81	5.03
One	4.34	4.58
Two	2.40	3.59
Three	0.94	3.06

Lactobacilli strains have higher proteolytic activity and acid tolerance compared to *Streptococcus thermophilus* (Shah and Jelen, 1990, Dave and Shah, 1998a, Dave and Shah, 1998b, Garault *et. al*., 2000). According to Gonzalez-Marquez *et. al*. (1997) the optimal pH growth for Lactic Acid Bacteria (LAB) is between 5.5 to 7.0, which explains the reduction of viable cells in control after subjection to acidic conditions at 120 minutes. According to Nadal *et. al*. (2010), the addition of whey proteins can improve the buffering capacity of a media, thus reducing the effect of acid environments for the bacterial strain.

3.2 Bile Tolerance

3.2.1 *Streptococcus thermophilus* ST-M5

Bile tolerance of *Streptococcus thermophilus* ST-M5 as influenced by the addition of whey protein isolate (WPI) over incubation during 5 hours is shown in Figure 3. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 5).

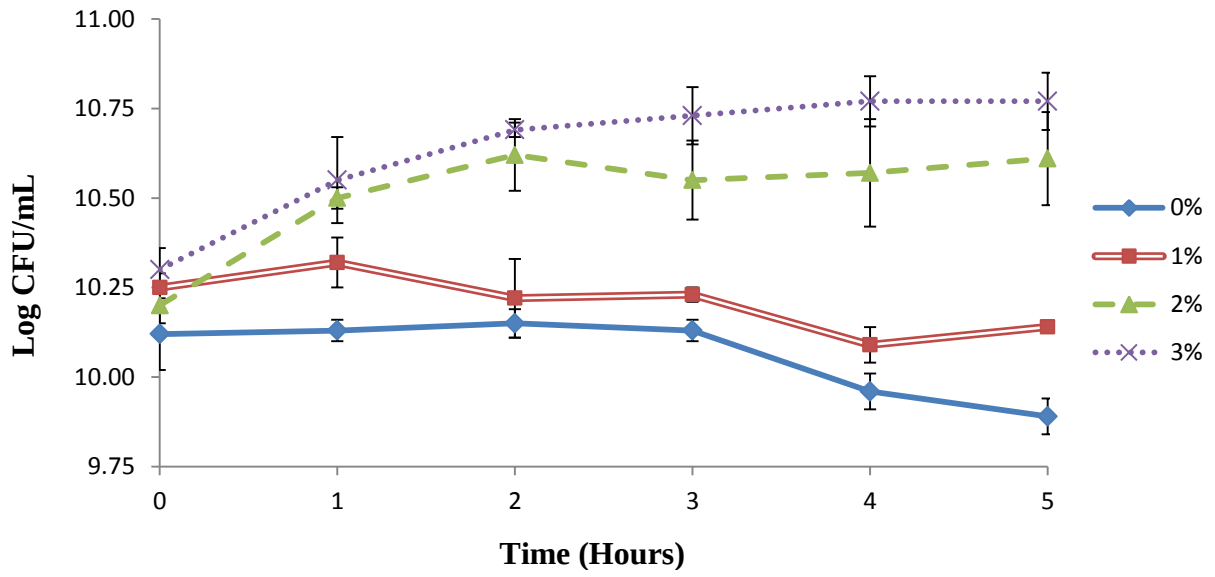

Figure 3. Bile tolerance of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 5 hours.

Addition of 2 and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to control and 1% WPI at 2, 3, 4 and 5 hours of incubation (Table 6). At 5 hours of incubation control showed significant (P<0.05) decrease in counts compared to its counts at hour 0 (Table 6). At 5 hours of incubation counts in 1% WPI were comparable to counts at hour 0 (Table 6). At 5 hours of incubation 2 and 3% WPI showed significantly (P<0.05) higher counts compared to their counts at hour 0 (Table 6).

Mean log difference (Table 7) in the counts of *Streptococcus thermophilus* ST-M5 was obtained by subtracting log CFU/mL of 5 hours from 0 hours of incubation. A positive number indicated bacterial death and negative numbers indicated bacterial growth. Bacterial death was observed among samples containing 0 and 1% WPI (Table 7). On the contrary, bacterial growth was observed among samples containing 2 and 3% WPI (Table 7). *Streptococcus thermophilus* ST-M5 showed a mean log difference of 0.23 and 0.11 CFU/mL in control and 1% WPI, respectively, compared to a mean log difference of -0.41 and -0.47 CFU/mL in 2 and 3% WPI, respectively (Table 7).

3.2.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

Bile tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by the addition of whey protein isolate (WPI) over incubation during 5 hours is shown in Figure 4. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 5). Use of 1, 2 and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to control at 3, 4, and 5 hours of incubation (Table 6). At 5 hours of incubation, 2 and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to control and 1% WPI (Table 6).

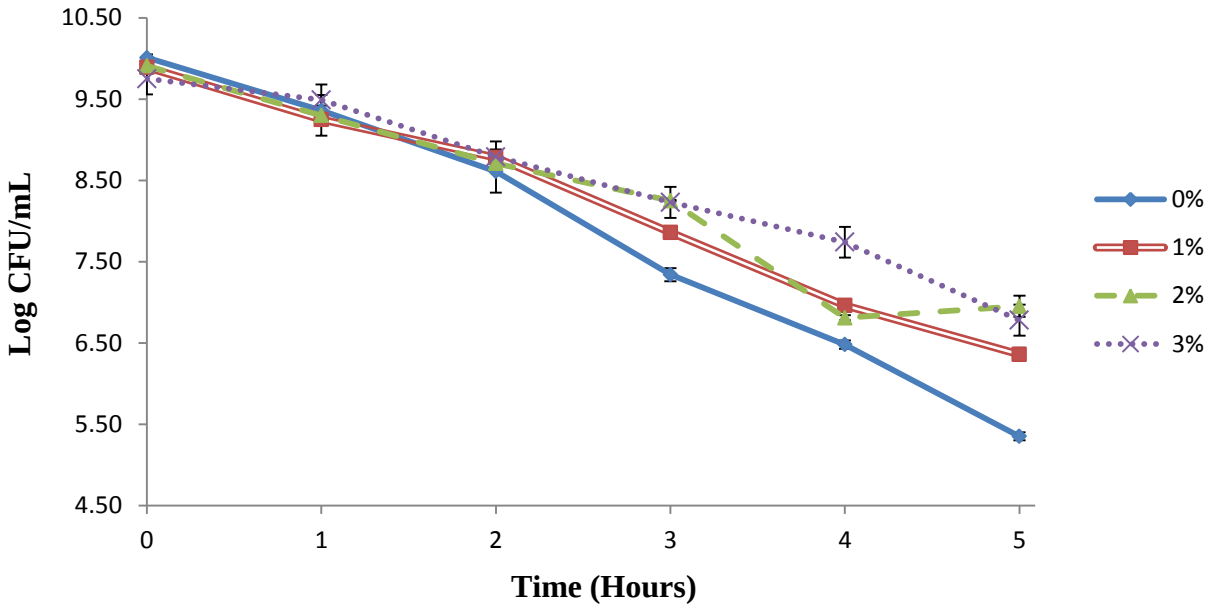

Figure 4. Bile tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 5 hours

Mean log difference (Table 7) in the counts of *Lactobacillus bulgaricus* LB-12 was obtained by subtracting log CFU/mL of 5 hours from 0 hours of incubation. A large number indicated higher bacterial death. All samples containing 1, 2 and 3% WPI showed lower bacteria death compared to control (Table 7). The bacterial death was the lower for 2 and 3% WPI compared to 0 and 1% WPI (Table 7).

Bile salts normally affect the survival of bacteria cells due to the high susceptibility of bacteria cell walls to bile presence (Jin *et. al*., 1998). According to Conway *et. al*. (1987), *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are not tolerant to acid environments (pH 3). A simulated small intestinal transit study by Charteris *et. al*. (1998) was conducted using 15 strains of potentially Lactobacilli and Bifidobacteria probiotics. They prepared solutions of sodium caseinate (0.1%) and WPI (0.1%) individually suspended in sterile saline solution (0.5% w/v).

The simulated small intestinal transit was conducted by mixing 1 mL pancreatic juice (0.1% pancreatin USP) 0.2 mL bacterium and 0.3 mL NaCl (control), 0.3 mL WPI solution, 0.3 mL sodium caseinate or a mix of both protein suspension in a 1:1 ratio. Charteris *et. al*. (1998) found an increase (1 log CFU/mL) in viable cells of *Lactobacillus* F19 compared to control at 4 hours of incubation.

Liong and Shah (2005) reported the bile tolerance for strains of *Lactobacillus acidophilus* and *Lactobacillus casei* at 2 hours of incubation in MRS broth containing 0.3% oxgall. They found the highest cell reduction in MRS broth (0.3% oxgall) compared to control (MRS broth). These results are in accordance with this study, where the presence of 2 and 3% WPI increased the survival of *Lactobacillus bulgaricus* LB-12 up to 1.6 log CFU/mL at 5 hours of exposure to bile salts compared to control (Table 7).

According to Charteris *et. al*. (1998) whey protein isolate can protect LAB from gastrointestinal stress by acting as a buffering agent and inhibiting activity of digestive enzyme.

Table 5. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate with the influence of bile (oxgall).

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	<0.0001
Hour	<0.0001	<0.0001
Treatment*Hour	<0.0001	<0.0001

Table 6. Least Square Means (Log CFU/mL) for bile tolerance of pure *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 5 hours.

Treatment	*Streptococcus thermophilus* ST-M5						*Lactobacillus bulgaricus* LB-12					
	Time (Hours)											
	0	1	2	3	4	5	0	1	2	3	4	5
Control	10.12^{EFG}	10.13^{EFG}	10.15^{EFG}	10.13^{EFG}	9.96^{GH}	9.89^{H}	10.01^{A}	9.36^{C}	8.61^{D}	7.34^{G}	6.48^{I}	5.35^{J}
One	10.25^{EF}	10.32^{DE}	10.22^{EF}	10.23^{EF}	10.09^{FGH}	10.14^{EFG}	9.89^{AB}	9.25^{C}	8.78^{D}	7.86^{F}	6.96^{H}	6.36^{I}
Two	10.20^{EF}	10.50^{CD}	10.62^{ABC}	10.55^{BC}	10.57^{ABC}	10.61^{ABC}	9.91^{AB}	9.30^{C}	8.71^{D}	8.25^{E}	6.81^{H}	6.95^{H}
Three	10.30^{DEF}	10.55^{ABC}	10.69^{ABC}	10.73^{AB}	10.77^{A}	10.77^{A}	9.75^{B}	9.49^{C}	8.79^{D}	8.23^{E}	7.74^{F}	6.78^{H}

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.

Table 7. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of bile (oxgall).

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
	Log CFU/mL	
Control	0.23	4.66
One	0.11	3.53
Two	-0.41	2.96
Three	-0.47	2.97

3.3 Growth

3.3.1 *Streptococcus thermophilus* ST-M5

Growth of *Streptococcus thermophilus* ST-M5 as influenced by the addition of whey protein isolate (WPI) over incubation during 60 hours is shown in Figure 5. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 8).

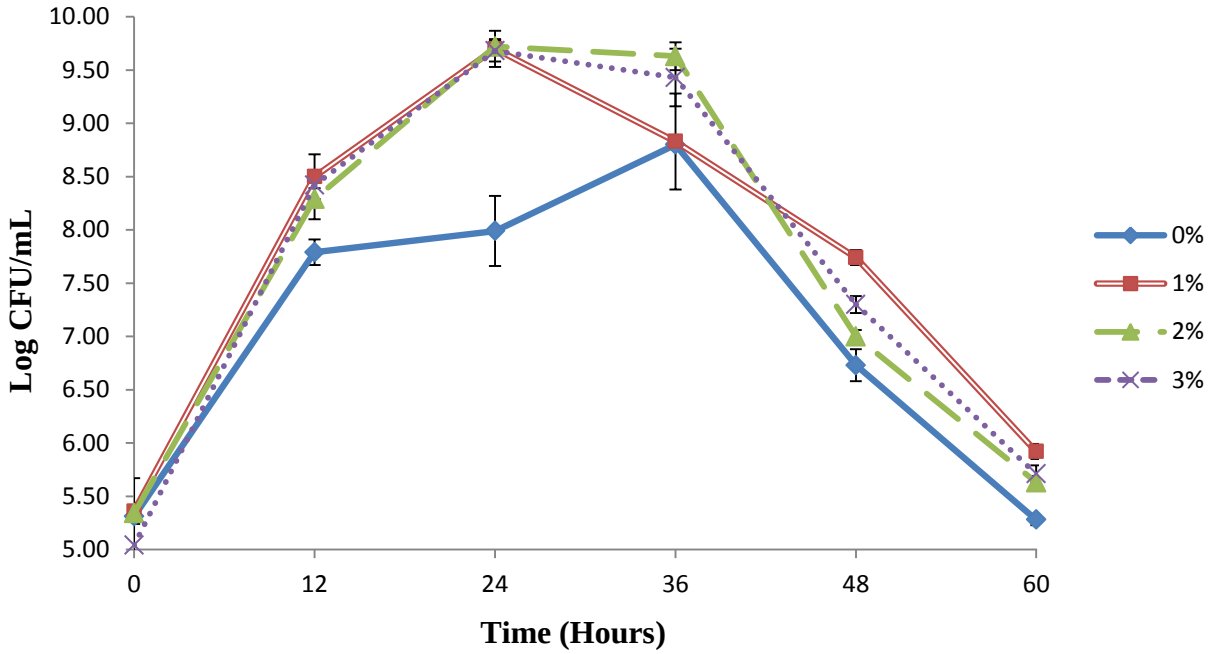

Figure 5. Growth of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 60 hours.

Use of 1 and 3% WPI showed significantly (P<0.05) higher growth compared to control at 12 hours of incubation (Table 9). Use of 1, 2 and 3% WPI showed significantly (P<0.05) higher growth compared to control at 24 hour of incubation (Table 9). At 36 hours of incubation 2 and 3% WPI showed significantly (P<0.05) higher growth compared to control (Table 9). At 48 and 60 hours of incubation 1% WPI showed significantly (P<0.05) higher growth compared to control (Table 9).

3.3.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

Growth of *Lactobacillus bulgaricus* LB-12 as influenced by the addition of whey protein isolate (WPI) over incubation during 60 hours is shown in Figure 6. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 8).

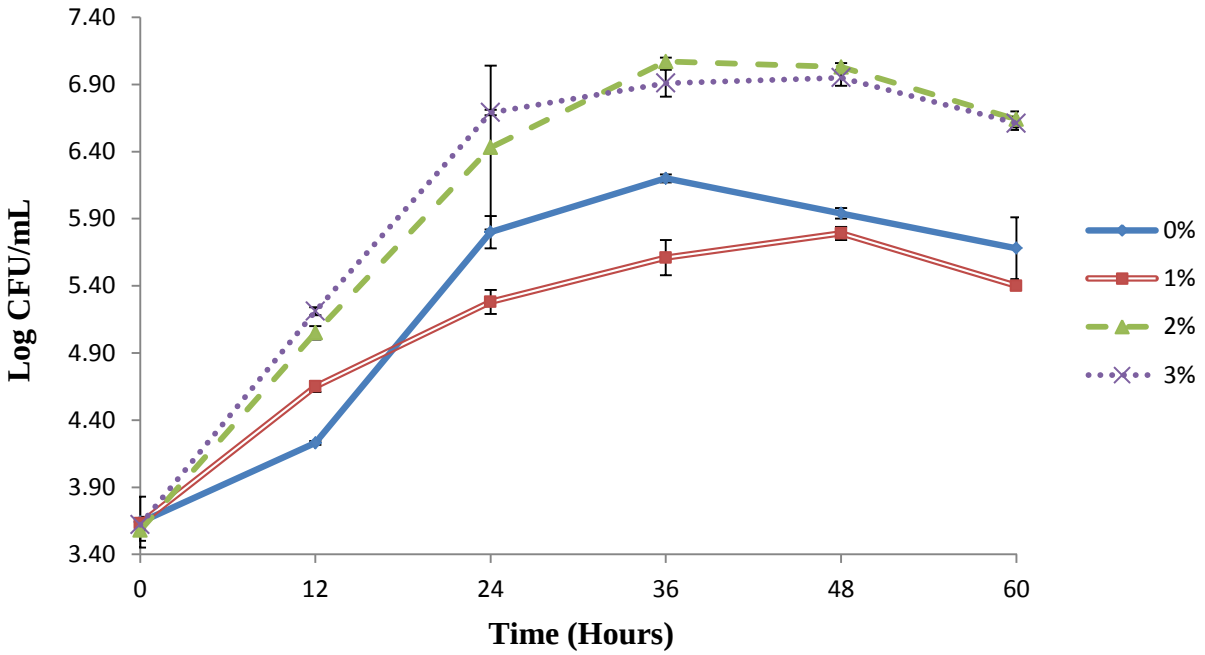

Figure 6. Growth of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 60 hours

At 24, 36, 48 and 60 hours of incubation of 2 and 3% w/v added whey protein isolate significantly increased the growth of *Lactobacillus bulgaricus* LB-12 (P<0.05) compared to control and 1% w/v added whey protein isolate (Table 9). Letort and Juillard (2001) reported that depletion of amino acids sources did not stop the growth of *Streptococcus thermophilus* but the growth rate was reduced by 1.8 log CFU/mL per hour. Strains of *Streptococcus thermophilus* require essential amino acids and peptides to grow (Garault *et. al*., 2000). *S. thermophilus* is less susceptible to depletion of amino acids compared to Lactobacilli strains (Garault *et. al*., 2000,

Letort and Juillard, 2001). However, the absence of Leucine, Isoleucine, Valine (Branched-chain amino acids), and Methionine can decrease the growth of *S. thermophilus* up to 50% (Garault *et. al*., 2000, Letort and Juillard, 2001). This is in accordance with the data shown in Table 9 were the addition of WPI at different levels, significantly increased the growth of *Streptococcus thermophilus* ST-M5 at 24, 36, 48, and 60 hours of incubation.

Despite peptides and amino acids provided by the addition of whey protein isolate, a decrease of viable counts of *Streptococcus thermophilus* ST-M5 at 60 hours of incubation was found in all samples, which can be due to metabolic process and lactic acid production of the bacterium that eventually decreased the pH in the media causing a reduction of viable counts of *S. thermophilus* (Shah and Jelen, 1990, Dave and Shah, 1998b, Garault *et. al*., 2000, Shah, 2007). Lactobacilli strains have a higher proteolytic activity compared to *S. thermophilus* (Dave and Shah, 1996, Dave and Shah, 1998a, Dave and Shah, 1998b, Garault *et. al*., 2000), thus making the growth of Lactobacilli responsible for changes in peptides levels. Addition of 2 and 3 % WPI significantly increased the growth of *Lactobacillus bulgaricus* LB-12 at 24, 36, 48, and 60 hours of incubation. This data is in accordance with Akalin *et. al*. (2007) which reported an increase of 1 log CFU/mL in the growth of *L. bulgaricus* in yogurt with 1.5% whey protein concentrate added compared to no whey protein concentrate addition. Besides providing peptides and amino acids, the addition of whey protein isolate acted as a buffer agent, preventing sudden changes in the acidity of the media and avoiding lethal pH levels for *L. bulgaricus* (Dave and Shah, 1998a, Dave and Shah, 1998b). This effect is showed in Table 9 where at 60 hours of incubation, samples containing 2 and 3% WPI showed significantly ($P<0.05$) higher growth of *L. bulgaricus* compare to control and 1% WPI.

Table 8. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate during 60 hours of incubation.

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	<0.0001
Hour	<0.0001	<0.0001
Treatment*Hour	<0.0001	<0.0001

Table 9. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 60 hours.

Treatment	*Streptococcus thermophilus* ST-M5						*Lactobacillus bulgaricus* LB-12					
	Time (Hours)											
	0	12	24	36	48	60	0	12	24	36	48	60
Control	5.31 ^{HIJ}	7.79 ^{EF}	7.99 ^{DE}	8.80 ^{C}	6.73 ^{G}	5.28 ^{IJ}	3.64 ^{K}	4.23 ^{J}	5.80 ^{DEF}	6.20 ^{CD}	5.94 ^{DE}	5.68 ^{EFG}
One	5.36 ^{HIJ}	8.50 ^{CD}	9.70 ^{A}	8.83 ^{BC}	7.74 ^{EF}	5.92 ^{H}	3.63 ^{K}	4.65 ^{IJ}	5.28 ^{GH}	5.61 ^{EFG}	5.79 ^{DEF}	5.40 ^{FGH}
Two	5.34 ^{HIJ}	8.29 ^{CDE}	9.72 ^{A}	9.63 ^{A}	7.00 ^{G}	5.63 ^{HIJ}	3.58 ^{K}	5.05 ^{HI}	6.43 ^{BC}	7.07 ^{A}	7.03 ^{A}	6.64 ^{ABC}
Three	5.04 ^{J}	8.42 ^{CD}	9.68 ^{A}	9.43 ^{AB}	7.30 ^{FG}	5.71 ^{HI}	3.62 ^{K}	5.21 ^{GH}	6.69 ^{ABC}	6.91 ^{AB}	6.95 ^{A}	6.61 ^{ABC}

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.

3.4 Protease activity

3.4.1 *Streptococcus thermophilus* ST-M5

Protease activity of *Streptococcus thermophilus* ST-M5 as influenced by the addition of whey protein isolate (WPI) over incubation during 24 hours is shown in Figure 7. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 10).

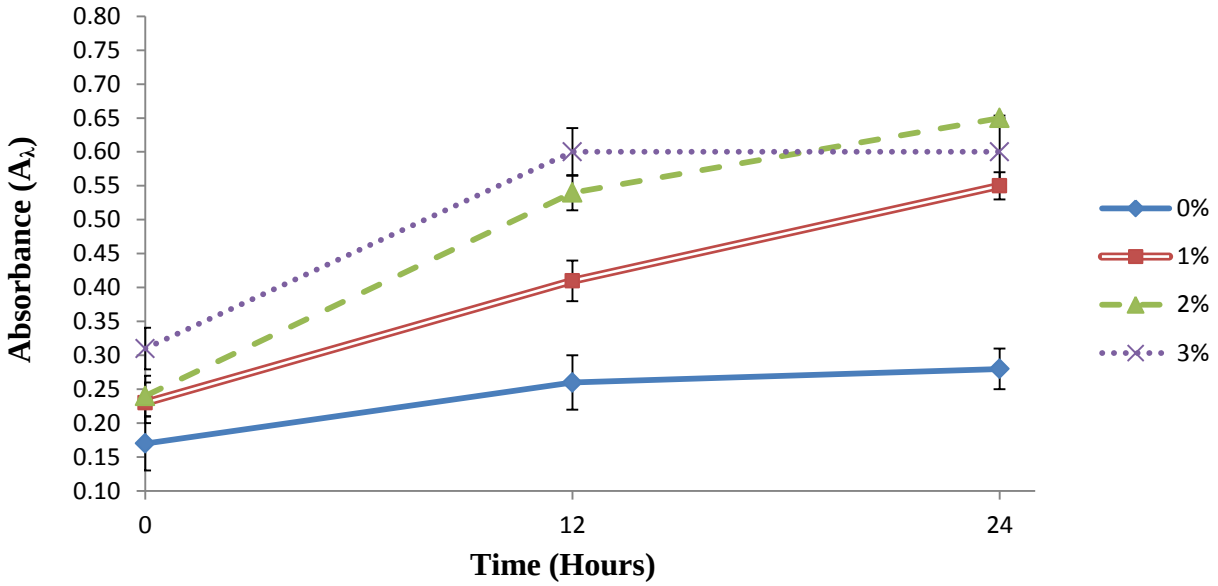

Figure 7. Protease activity of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentration over the incubation period of 24 hours.

Use of 1, 2 and 3%WPI showed significantly (P<0.05) higher protease activity compared to control at 12 and 24 hours of incubation (Table 11). At 12 hours of incubation use of 2 and 3% WPI significantly (P<0.05) increased protease activity of *Streptococcus thermophilus* ST-M5 compared to control and 1% WPI (Table 11). At 24 hours of incubation all samples (0, 1, 2 and 3% WPI) showed significantly (P<0.05) higher protease activity compared to their respective hour 0 (Table 11). The increase rate of protease activity was higher when 1, 2 and 3% w/v whey protein isolate was added (Table 11).

3.4.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

Protease activity of *Lactobacillus bulgaricus* LB-12 as influenced by the addition of whey protein isolate (WPI) over incubation during 24 hours is shown in Figure 8. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 7).

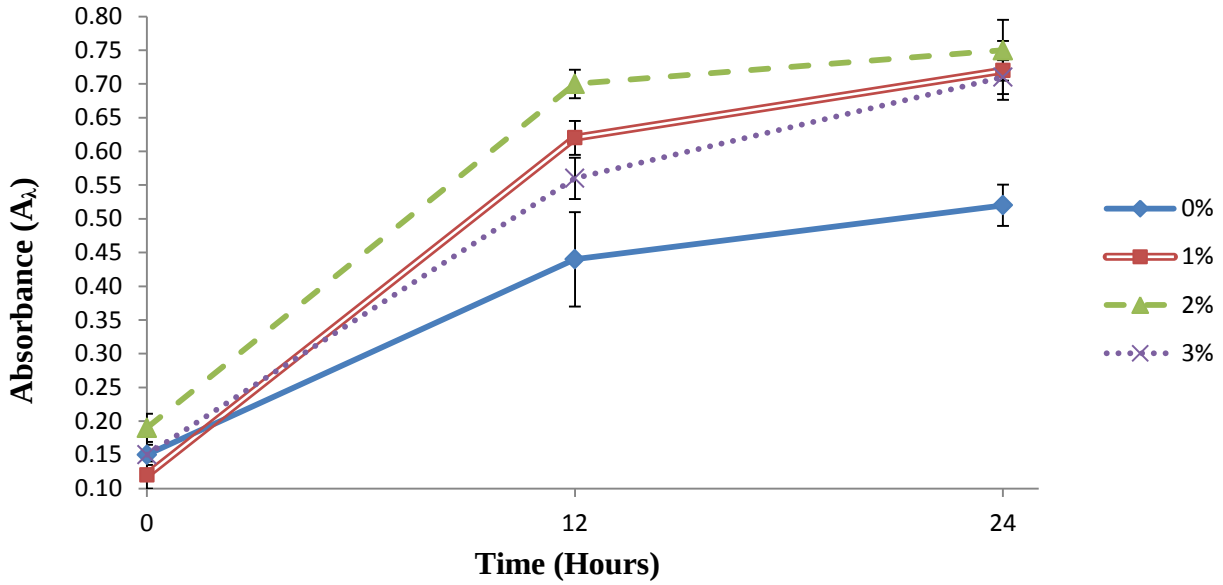

Figure 8. Protease activity of *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration over the incubation period of 60 hours.

Use of 1, 2 and 3% WPI showed significantly (P<0.05) higher protease activity compared to control at 12 and 24 hours of incubation (Table 11). At 12 hours of incubation addition of 1 and 2% WPI significantly (P<0.05) increased protease activity of *Streptococcus thermophilus* ST-M5 compared to control (Table 11). A significantly (P<0.05) increase of protease activity of *L. bulgaricus* and *S. thermophilus* was observed at 24 hours of incubation in all samples (0, 1, 2 and 3% WPI) compared to their respective hour 0 (Table 11). At 24 hours of incubation all samples (0, 1, 2 and 3% WPI) inoculated with *Lactobacillus bulgaricus* LB-12 showed higher protease activity than samples inoculated with *Streptococcus thermophilus* ST-M5 (Table 11). Protease

activity of control inoculated with *S. thermophilus* ST-M5 showed lower increase at 24 hours of incubation compared to protease activity of control inoculated with *L. bulgaricus* LB-12 (Table 11).

Use of co-cultures containing strains of *L. bulgaricus* and *S. thermophilus* is common in the manufacture of fermented dairy products (Almeida *et. al*., 2009) because both bacteria grow in a synergistic way (Sieuwerts *et. al*., 2010). *S. thermophilus* produces folic acid that stimulates the growth of *L. bulgaricus*, which metabolize proteins to peptides and free amino acids that stimulates the growth of *S. thermophilus* (Sieuwerts *et. al*., 2010). Higher protease activity of *L. bulgaricus* compared to *S. thermophilus* was observed in this study, which is in accordance with Shah and Jelen, (1990), Dave and Shah, (1998a), Dave and Shah, (1998b) and Garault *et. al*., (2000). An increase of protease activity of both bacteria was observed when whey protein isolate was added. Addition of whey protein isolate increased availability of free amino acids and peptides for the bacteria (Siso, 1996, de Wit, 1998, Madureira *et. al*., 2007) and increased the protease activity rate over 24 hours of incubation.

Table 10. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 protease activity (absorbance) in the presence of 0, 1, 2 and 3% w/v of added whey protein isolate during 24 hours of incubation.

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	<0.0001
Hour	<0.0001	<0.0001
Treatment*Hour	<0.0001	<0.0001

Table 11. Least Square Means (Absorbance) for protease activity of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate over the incubation period of 24 hours.

Treatment	*Streptococcus thermophilus* ST-M5			*Lactobacillus bulgaricus* LB-12		
	Time (Hours)					
	0	12	24	0	12	24
Control	0.17^{E}	0.26^{DE}	0.28^{D}	0.15^{E}	0.44^{D}	0.52^{CD}
One	0.23^{DE}	0.41^{C}	0.55^{AB}	0.12^{E}	0.62^{BC}	0.72^{A}
Two	0.24^{DE}	0.54^{B}	0.65^{A}	0.19^{E}	0.70^{AB}	0.75^{A}
Three	0.31^{D}	0.60^{AB}	0.60^{AB}	0.15^{E}	0.56^{C}	0.71^{AB}

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.

SECTION 2: Yogurt Analysis

3.5 Acid Tolerance

3.5.1 *Streptococcus thermophilus* ST-M5

The acid tolerance of *Streptococcus thermophilus* ST-M5 from a manufactured fat free plain yogurt (at 7 days of storage) as influenced by added whey protein isolate concentration (WPI) over 120 minutes of incubation is shown in Figure 9. Treatment*minutes interaction effect, treatment effect and minutes effect were significant (P<0.05) (Table 12).

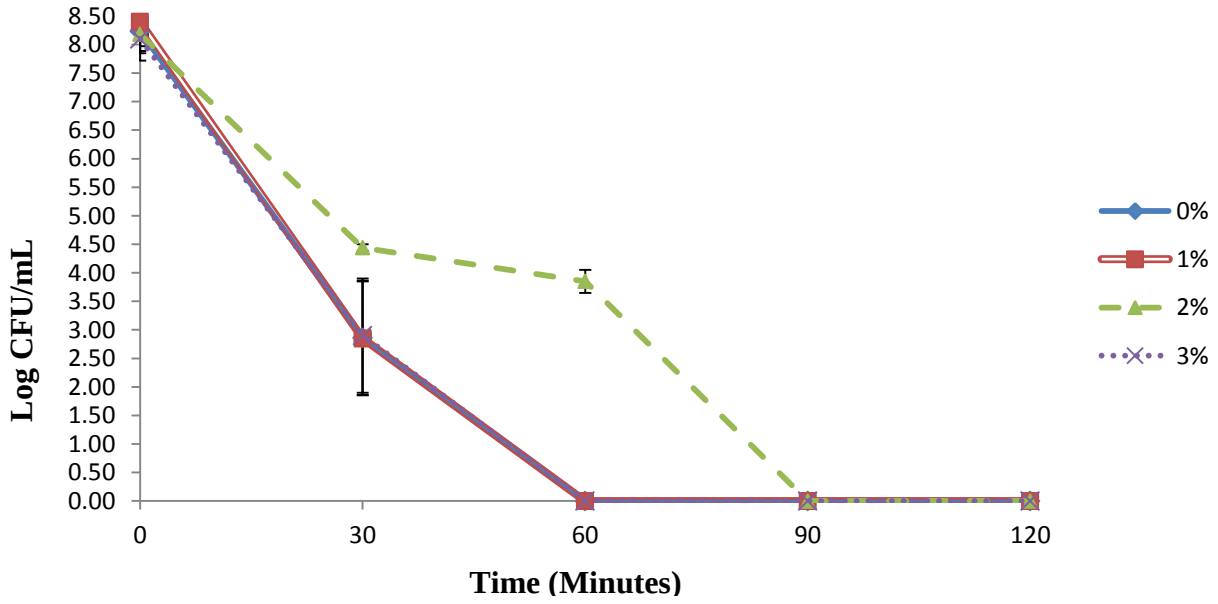

Figure 9. Acid tolerance of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 120 minutes.

Addition of 2% WPI showed significantly (P<0.05) higher viable cell counts compared to 0, 1 and 3% WPI at 30 minutes of incubation (Table 13). Use of 2% WPI showed viable cells of *Streptococcus thermophilus* ST-M5 compared to no detection of *S. thermophilus* cells on 0, 1 and 3% WPI at 60 minutes of incubation. (Table 13). At 90 minutes of 0, 1, 2 and 3 % WPI was

unable to survive. Mean log difference (Table 14) in the counts of *Streptococcus thermophilus* ST-M5 was obtained by subtracting log CFU/mL of 30 minutes from 0 minutes of incubation. A low number indicated lower bacterial death. The bacterial death was the lowest for 2% w/v added whey protein isolate compared to the rest (Table 14).

3.5.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

The acid tolerance of *Lactobacillus bulgaricus* LB-12 from a manufactured fat free plain yogurt (at 7 days of storage) as influenced by added whey protein isolate concentration (WPI) over 120 minutes of incubation is shown in Figure 10. Treatment*minutes interaction effect, treatment effect and minutes effect were significant (P<0.05) (Table 12).

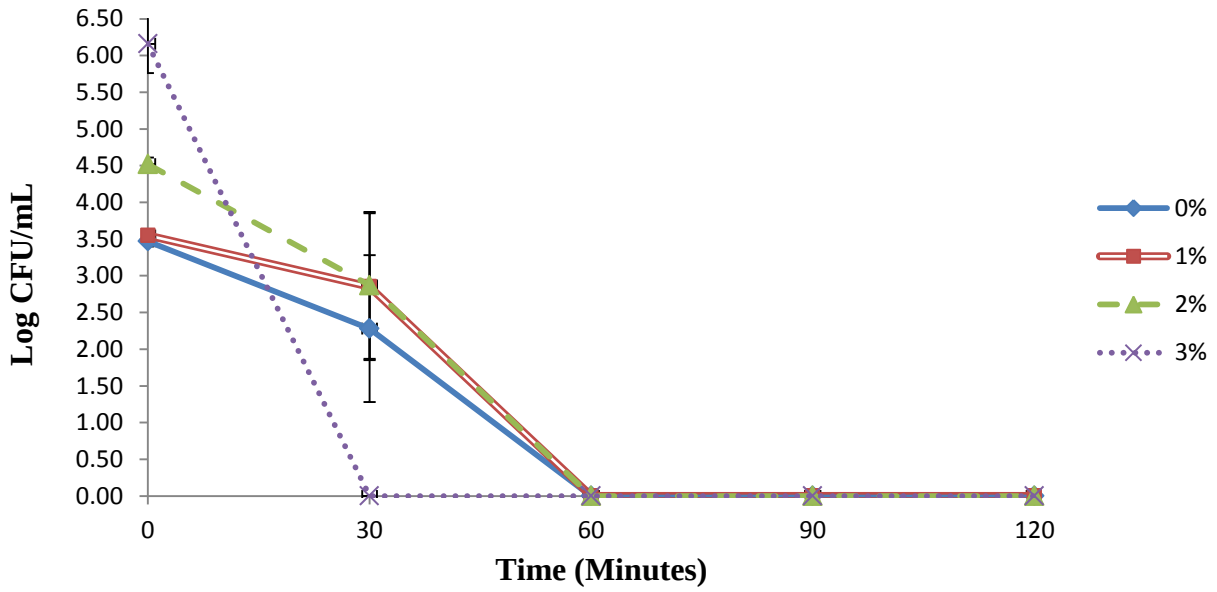

Figure 10. Acid tolerance of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 120 minutes.

At 30 minutes of incubation no significantly (P>0.05) differences were found among all samples

(0, 1, 2 and 3% WPI) (Table 13). At 60 minutes of incubation, no viable counts of *Lactobacillus bulgaricus* LB-12 were found in any of the samples (Table 13).

Table 12. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 viable cell counts from fat free plain yogurt containing 0, 1, 2 and 3% w/v of added whey protein isolate under the influence of acidic broth.

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	0.0170
Minutes	<0.0001	<0.0001
Treatment*Minutes	0.0003	<0.0001

Mean log difference (Table 14) in the viable counts of *Lactobacillus bulgaricus* LB-12 was obtained by subtracting log CFU/mL of 30 minutes from 0 minutes of incubation period. A low number indicated low bacterial death. Bacterial death was lowest for 1 % WPI at 30 minutes of incubation. *S. thermophilus* and *L. bulgaricus* do not have good tolerance to acidic environments compared to strains of *L. casei*, *L. acidophilus and* Bifidobacteria (Charteris *et. al*., 1998). The control *L. bulgaricus* showed a lower mean log difference (1.19 log CFU/mL) compared to the control *S. thermophilus* (5.37 log CFU/mL) which can indicate more resistance of *L. bulgaricus* compared to *S. thermophilus* (Table 14). Same behavior is mentioned by Shah and Jelen (1990).

When acid tolerance was evaluated on pure culture bacteria, WPI was directly added to broths (pH 2), then inoculated and incubated and significantly higher viable counts of *S. thermophilus* and *L. bulgaricus* were found, which is an indicative that whey protein isolate served as a buffer as proposed by Nadal *et. al*. (2010). According to Lee and Vickers (2008) addition of whey proteins to beverages requires higher amounts of acid to reach a specific acidic pH (below 4.5) compared to beverages without whey proteins.

Cell walls of starter culture bacteria contain proteinases and peptidases (Salminen *et. al*., 2004). Polypeptides are generated from milk proteins by the action of proteinases and peptidases present in starter culture bacteria cell walls (Salminen *et. al*., 2004). The action of peptidases from cell walls provides peptides and free amino acids used by starter culture bacteria for metabolic processes (Garault *et. al*., 2000, Güler-Akin *et. al*., 2009, Salminen *et. al*., 2004). The addition of whey protein isolate to yogurt mixes can increase the ammount of protein in the yogurt. A study by Pescuma *et. al*., (2007) evaluated the ability of *Streptococcus thermophilus* CRL 804 and *Lactobacillus bulgaricus* CRL 454 to hydrolyze whey proteins (WPC 89% protein) in a media. According to Pescuma *et. al*., (2007), *S. thermophilus* and *L. bulgaricus* were capable to break down up to 21% of β-lactoglobulin (β-LG) and 26% of α-lactalbumin (α-LA) into smaller peptides (7 kDa) and free amino acids. This indicates that the enzymes present in the cell walls of starter culture bacteria have an effect on whey proteins.

At 120 minutes of acid exposure, use of 1, 2 and 3% w/v WPI increased the acid tolerance of pure *S. thermophilus* up to 5 log CFU/mL and that of *L. bulgaricus* up to 1.75 log CFU/mL compared to control. In contrast, at 60 minutes of acid exposure, in yogurts containing 2% added WPI, the acid tolerance test counts of *S. thermophilus* increased by 3.85 CFU/mL and that of *L. bulgaricus* increased by 0.5 log CFU/mL compared to control (Table 13). No viable cells were found after 60 minutes of acid exposure. These changes between acid tolerance of pure culture bacteria and acid tolerance of bacteria from yogurt can be due to differences in buffer capacity of WPI added to acidified broths and incubated for 2 hours compared to the same WPI levels added to yogurt mixes that were under the influence of metabolic process of culture bacteria during the 7 days of storage.

Table 13. Least Square Means (Log CFU/mL) for acid tolerance of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt containing 0, 1, 2 and 3% w/v of added whey protein isolate over the incubation period of 120 minutes at pH 2.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5					*Lactobacillus bulgaricus* LB-12				
	Time (Minutes)									
	0	30	60	90	120	0	30	60	90	120
Control	8.23 ^{A}	2.86 ^{CD}	ND	ND	ND	3.47 ^{BC}	2.28 ^{DE}	ND	ND	ND
One	8.40 ^{A}	2.85 ^{CD}	ND	ND	ND	3.55 ^{BC}	2.85 ^{CDE}	ND	ND	ND
Two	8.18 ^{A}	4.44 ^{B}	3.85 ^{BC}	ND	ND	4.52 ^{B}	2.87 ^{CD}	ND	ND	ND
Three	8.10 ^{A}	2.90 ^{CD}	ND	ND	ND	6.16 ^{A}	ND	ND	ND	ND

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.
ND Counts were not detected at lowest possible dilution.

Table 14. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration in the presence of acid.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
	Log CFU/mL	
Control	5.37	1.19
One	5.55	0.70
Two	3.74	1.65
Three	5.20	6.16*

* Counts were not detected at lowest possible dilution.

3.6 Bile Tolerance

3.6.1 *Streptococcus thermophilus* ST-M5

The bile tolerance of *Streptococcus thermophilus* ST-M5 from a manufactured fat free plain yogurt (after 7 days of storage) as influenced by added whey protein isolate concentration (WPI) over 5 hours of incubation is shown in Figure 11. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 15).

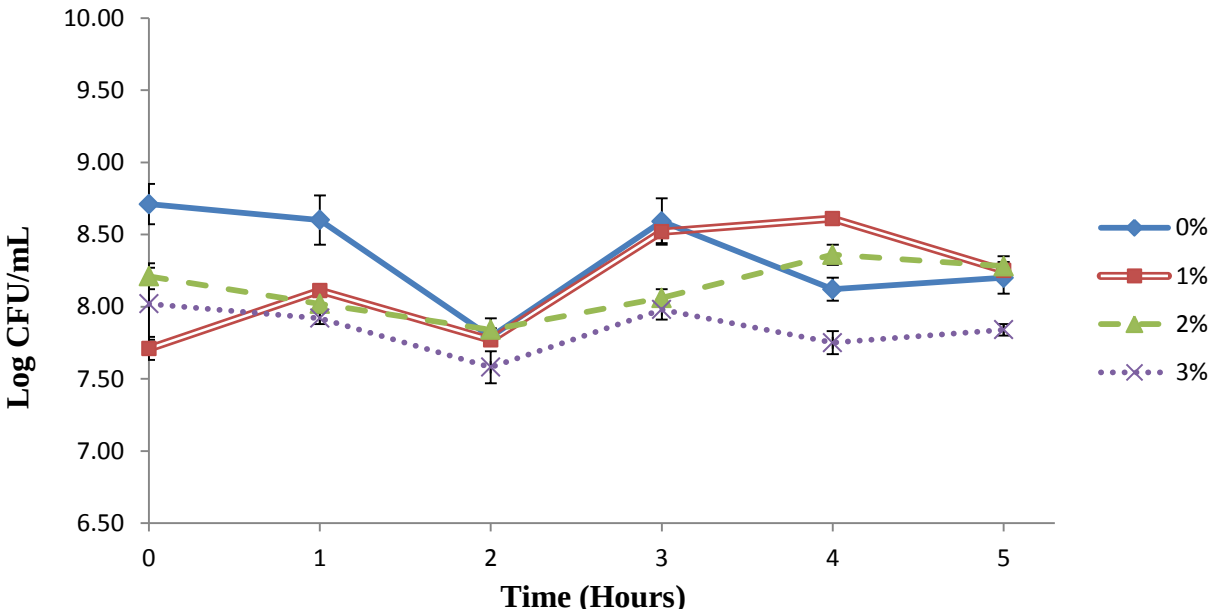

Figure 11. Bile tolerance of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 5 hours.

Addition of 0, 1 and 2% WPI showed significantly (P>0.05) higher viable cell counts compared to 3% WPI at 4 and 5 hours of incubation (Table 16). At 5 hours of incubation yogurts containing 0% WPI showed significantly (P<0.05) lower counts compared to its counts at hour 0 (Table 16). At 5 hours of incubation yogurts containing 1% WPI showed significantly (P<0.05) higher counts compared to its counts at hour 0 (Table 16).

Mean log difference (Table 17) in the counts of *Streptococcus thermophilus* ST-M5 was obtained by subtracting log CFU/mL of 5 hours from 0 hours of incubation. A positive low number indicated bacterial death and negative numbers indicated bacterial growth. Bacterial death was observed in samples containing 0% WPI (Table 17). On the contrary, bacterial growth was observed for 1% WPI (Table 17). *Streptococcus thermophilus* ST-M5 showed a mean log difference of 0.12 CFU/mL in 0% WPI compared to a mean log difference of -0.54 CFU/mL in 1% WPI (Table 17).

3.6.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

The bile tolerance of *Lactobacillus bulgaricus* LB-12 from a fat free plain yogurt (after 7 days of storage) as influenced by added whey protein isolate concentration (WPI) over 5 hours of incubation is shown in Figure 12. Treatment*hour interaction effect, treatment effect and hour effect were significant (P<0.05) (Table 15).

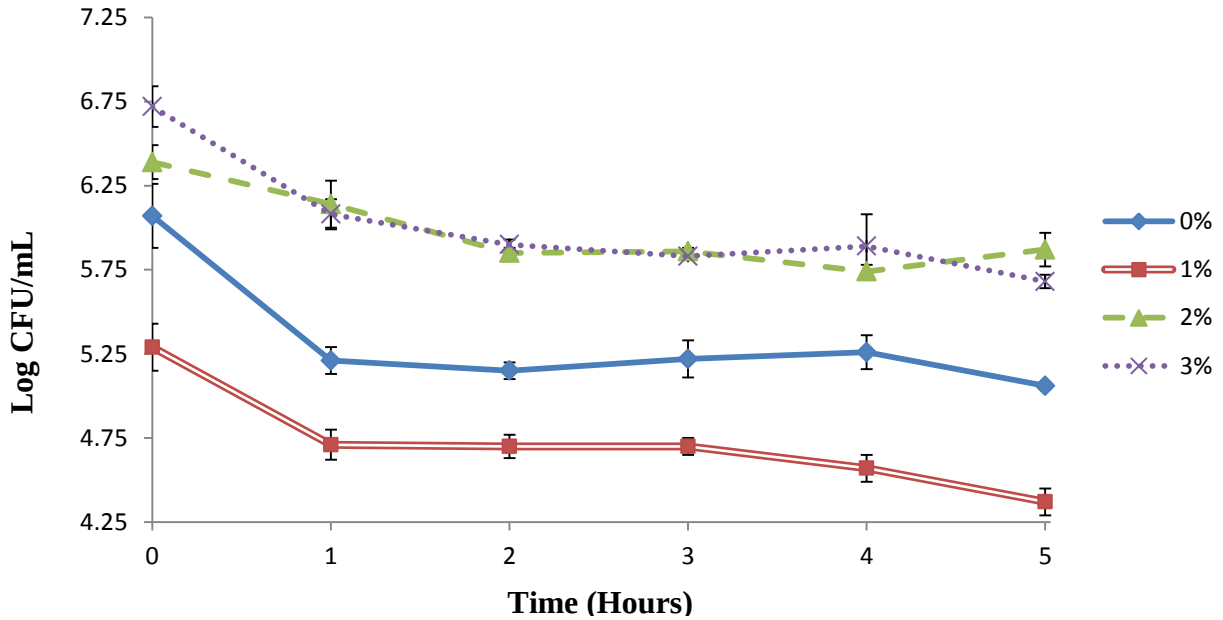

Figure 12. Bile tolerance of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt after 7 days of storage as influenced by added whey protein isolate concentration over the incubation period of 5 hours.

Addition of 2 and 3% WPI showed significantly (P<0.05) higher viable cell counts compared to 0 and 1% WPI at 1, 2, 3, 4 and 5 hours of incubation (Table 16). At 5 hours all samples showed significantly (P<0.05) lower counts compared to their counts at hour 0 (Table 16).

Table 15. Probability > F Value (Pr > F) for fixed effects of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts in the presence of 0, 1, 2 and 3% w/w of added whey protein isolate with the influence of bile (oxgall).

Effect	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
Treatment	<0.0001	<0.0001
Hour	<0.0001	<0.0001
Treatment*Hour	<0.0001	<0.0001

Mean log difference is shown in Table 17. A higher number indicated higher bacterial death. Yogurts containing 2% WPI showed lower bacteria death compared to the rest (Table 17). Differences in the initial count (hour 0) of *Lactobacillus bulgaricus* LB-12 (Table 16) are caused by a growth increase during 7 days of storage. Addition of whey protein isolate increased the activity of *Lactobacillus bulgaricus* LB-12. This increase can be caused by the higher proteolytic activity of Lactobacilli strains (Dave and Shah, 1998a, Dave and Shah, 1998b, Garault *et. al*., 2000). At 5 hours all yogurts containing 0, 1, 2 and 3% WPI showed significantly lower viable cell counts (P<0.05) compared to hour 0 (Table 16). Decrease of counts is normal because the susceptibility of phospholipid cell walls of bacteria to bile salts (Jin *et. al*., 1998). Bile works as a detergent that emulsifies and solubilizes lipids (Begley *et. al*., 2005). Higher concentration of bile dissolve lipids present in the phospholipid cell walls of bacteria, making bacteria susceptibility in the ion exchange transport affecting acid adaptation of bacteria and causing shrunken and leakage of intracellular material and eventually bacterial death (Begley *et. al*., 2005).

Table 16. Least Square Means (Log CFU/mL) for bile tolerance of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt containing 0, 1, 2 and 3% w/w of added whey protein isolate as influenced by bile (oxgall) over the incubation period of 5 hours.

Treatment	*Streptococcus thermophilus* ST-M5						*Lactobacillus bulgaricus* LB-12					
	Time (Hours)											
	0	1	2	3	4	5	0	1	2	3	4	5
Control	8.71 ^{A}	8.60 ^{AB}	7.79 ^{IJKL}	8.59 ^{AB}	8.12 ^{DEFGH}	8.20 ^{DEFG}	6.07 ^{CD}	5.21 ^{F}	5.15 ^{F}	5.22 ^{F}	5.26 ^{F}	5.06 ^{F}
One	7.71 ^{KL}	8.11 ^{DEFGH}	7.77 ^{IJKL}	8.52 ^{ABC}	8.61 ^{AB}	8.25 ^{CDEF}	5.29 ^{F}	4.71 ^{G}	4.70 ^{G}	4.70 ^{G}	4.57 ^{GH}	4.37 ^{H}
Two	8.21 ^{DEFG}	8.02 ^{EFGHIJ}	7.84 ^{HIJKL}	8.06 ^{EFGHI}	8.36 ^{BCD}	8.28 ^{CDE}	6.39 ^{B}	6.14 ^{BC}	5.85 ^{CDE}	5.86 ^{CDE}	5.74 ^{E}	5.87 ^{CDE}
Three	8.02 ^{EFGHIJ}	7.92 ^{GHIJK}	7.58 ^{L}	7.98 ^{FGHIJK}	7.75 ^{JKL}	7.84 ^{HIJKL}	6.72 ^{A}	6.08 ^{CD}	5.90 ^{CDE}	5.83 ^{DE}	5.89 ^{CDE}	5.68 ^{E}

^{ABC} LSMeans with different letter within the table for a microorganism are significantly different.

Table 17. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt as influenced by added whey protein isolate concentration in the presence of bile (oxgall).

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
	Log CFU/mL	
Control	0.51	1.01
One	-0.54	0.92
Two	-0.07	0.52
Three	-0.18	1.04

Positive number indicates bacterial death while negative numbers indicates bacterial growth.

3.7 Growth

3.7.1 *Streptococcus thermophilus* ST-M5

The growth of *Streptococcus thermophilus* ST-M5 in the manufactured fat free plain yogurt as influenced by added WPI over storage of 35 days is shown in Figure 13. Treatment*day interaction effect was not significant ($P>0.05$) while the treatment effect and day effect were significant ($P<0.05$) (Table 18).

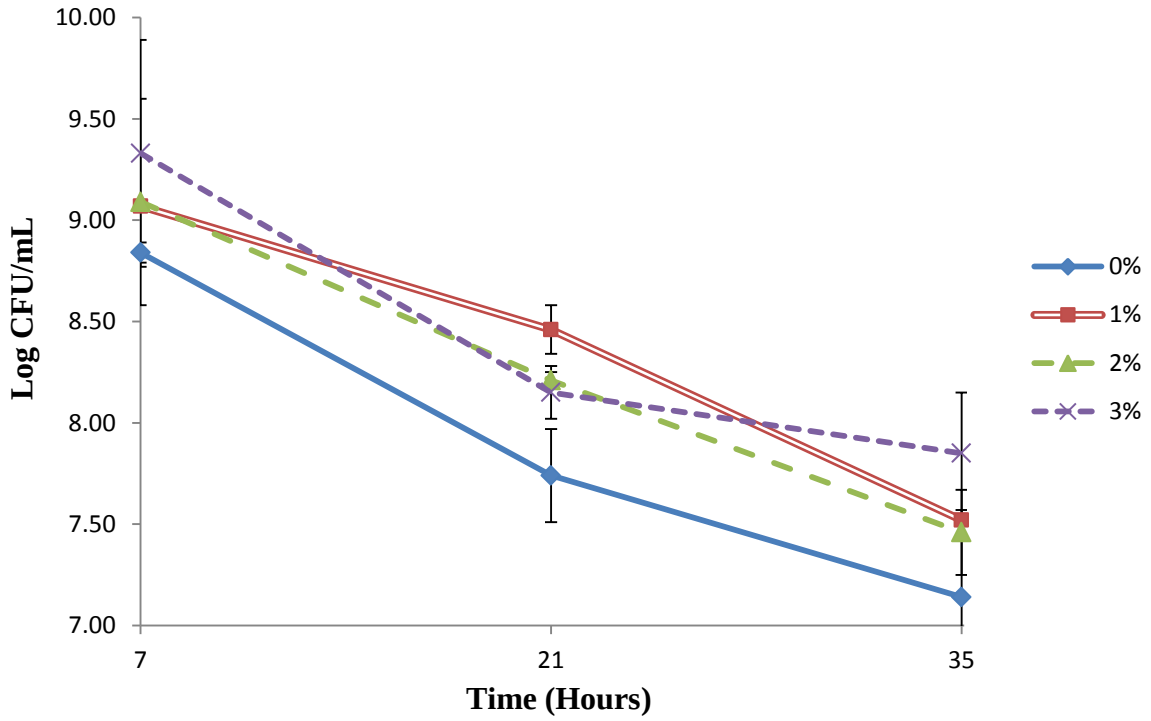

Figure 13. Growth of *Streptococcus thermophilus* ST-M5 from fat free plain yogurt as influenced by added whey protein isolate concentration over storage period of 35 days.

Addition of 1 and 3% WPI showed higher counts compared to control (Table 19). Significant ($P<0.05$) decrease was observed at 21 and 35 days of storage compared to day 7 (Table 20).

Table 18. Probability > F Value (Pr > F) for fixed effects of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 counts, *Streptococcus thermophilus* ST-M5 counts, apparent viscosity, pH, titratable acidity and syneresis of yogurts containing 0, 1, 2 and 3% w/v of added whey protein isolate over storage period of 35 days.

Effect	*S. thermophilus* ST-M5	*L. bulgaricus* LB-12	pH	TA	Apparent Viscosity	Syneresis
Treatment	0.0043	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Day	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Treatment*Day	0.5165	<0.0001	0.1661	0.0203	0.0005	0.0089

TA = Titratable Acidity.

Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 (Table 22) was obtained by subtracting log CFU/mL of day 35 from day 7 of storage. A low number indicated low bacterial death. Lower bacterial was observed for 1, 2 and 3% WPI samples compared to control. Bacterial death was the lowest for 3% WPI compared to the rest (Table 22).

Akalin *et. al*. (2007) studied the effect of whey protein concentrate 80% protein (WPC) addition to a reduced fat yogurt mix (1.5% fat). They reported counts of reduced fat yogurt containing 1.5% WPC significantly higher than counts of reduced fat yogurt without added WPC (8.56 log CFU/mL and 8.08 log CFU/mL respectively) at the end of 28 days of storage. According to Akalin *et.al.* (2007) WPC are rich in β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). When whey proteins are added to yogurt mixes, high temperatures during processing increases the availability of amino acids and peptides required for Lactic Acid Bacteria (LAB) growth (Akalin *et. al*., 2007). Besides the addition of peptides and

amino acids to be used by LAB, addition of whey protein can affect the pH of growth media (Nadal *et. al*., 2010).

Table 19. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 as influenced by added whey protein isolate concentrations.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5
	LS Means
Control	7.91 [b]
One	8.35 [a]
Two	8.25[ab]
Three	8.44 [a]

[ab] LSMeans with different letter within the column are significantly different.

Table 20. Least Square Means (Log CFU/mL) for growth of *Streptococcus thermophilus* ST-M5 as influenced by the storage period of 35 days.

Storage Period (Days)	*Streptococcus thermophilus* ST-M5
	LS Means
7	9.08[a]
21	8.14[b]
35	7.49[c]

[ab] LSMeans with different letter within the column are significantly different.

3.7.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

The growth of *Lactobacillus bulgaricus* LB-12 in the manufactured fat free plain yogurt as influenced by added WPI over storage of 35 days is shown in Figure 14. Treatment*day interaction effect, treatment effect and day effect were significant (P<0.05) (Table 18).

Addition of 3% WPI showed significantly (P<0.05) higher counts than 0, 1 and 2% WPI at 7, 21 and 35 days of storage (Table 21). At 35 days no viable counts were observed in

control compared to 3.64, 3.69 and 5.03 log CFU/mL in yogurts containing 1, 2 and 3% WPI, respectively (Table 21). A decrease in viable counts was observed in all treatments at day 35 of storage compared to their counts at day 7 (Table 21). Similar behavior was reported by Marafon *et. al*. (2011) and Akalin *et. al*. (2007) who reported that the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* in yogurt declined during storage period of 28 days.

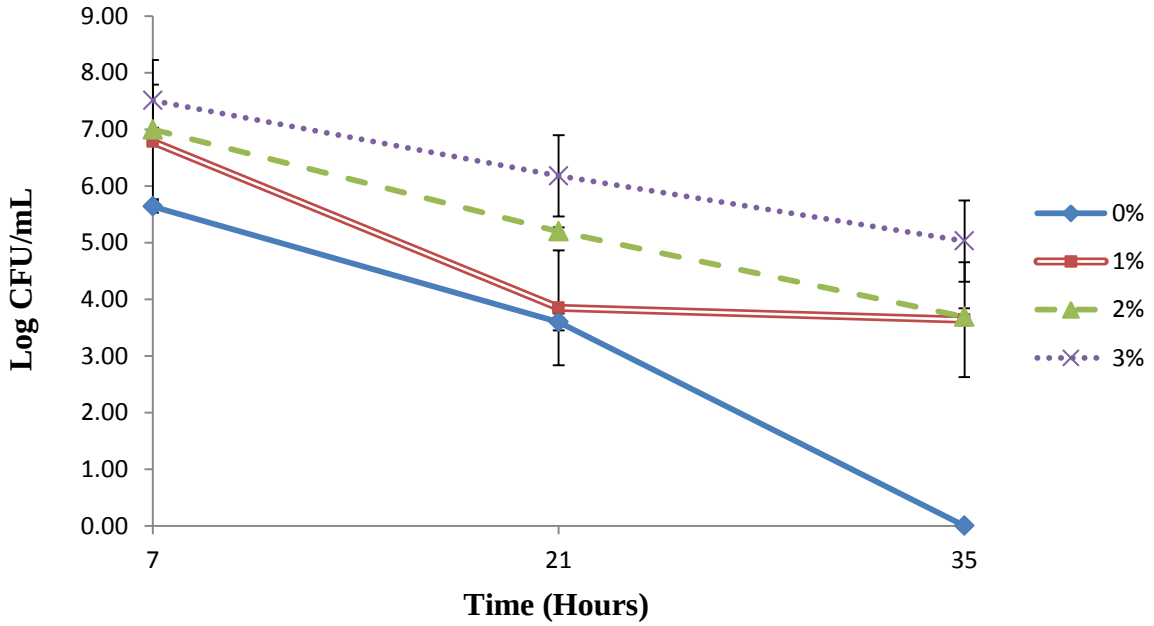

Figure 14. Growth of *Lactobacillus bulgaricus* LB-12 from fat free plain yogurt as influenced by added whey protein isolate concentration over storage period of 35 days.

Mean log difference in the viable counts of *Lactobacillus bulgaricus* LB-12 (Table 22) was obtained by subtracting log CFU/mL of day 35 from day 7 of storage. A low number indicated lower bacterial death. Bacterial death was lower in yogurts containing 1, 2 and 3% WPI compared to control (Table 22). Bacterial death was the lowest for 3% WPI showed the lowest compared to the rest (Table 22).

Table 21. Least Square Means (Log CFU/mL) growth of pure *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein over the incubation period of 12 hours.

Treatment	*Lactobacillus bulgaricus* LB-12		
	Storage Time (Days)		
	7	21	35
Control	5.64 ^{D}	3.60 ^{F}	ND
One	6.78 ^{B}	3.85 ^{F}	3.64 ^{F}
Two	7.00 ^{B}	5.20 ^{E}	3.69 ^{F}
Three	7.51 ^{A}	6.18 ^{C}	5.03 ^{E}

^{ABC} LSMeans with different letter within the table are significantly different.
ND Counts were not detected at lowest possible dilution.

Table 22. Mean log difference in the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 as influenced by added whey protein isolate concentration.

Added Whey Protein Isolate Concentration (%)	*Streptococcus thermophilus* ST-M5	*Lactobacillus bulgaricus* LB-12
	Log CFU/mL	
Control	1.70	5.64*
One	1.55	3.14
Two	1.63	3.31
Three	1.48	2.48

*Counts were not detected at lowest possible dilution.

3.8 pH

The pH of yogurts as influenced by added WPI over storage of 35 days is shown in Figure 15. Treatment*day interaction effect was not significant (P>0.05) while treatment effect and day effect were significant (P<0.05) (Table 18).

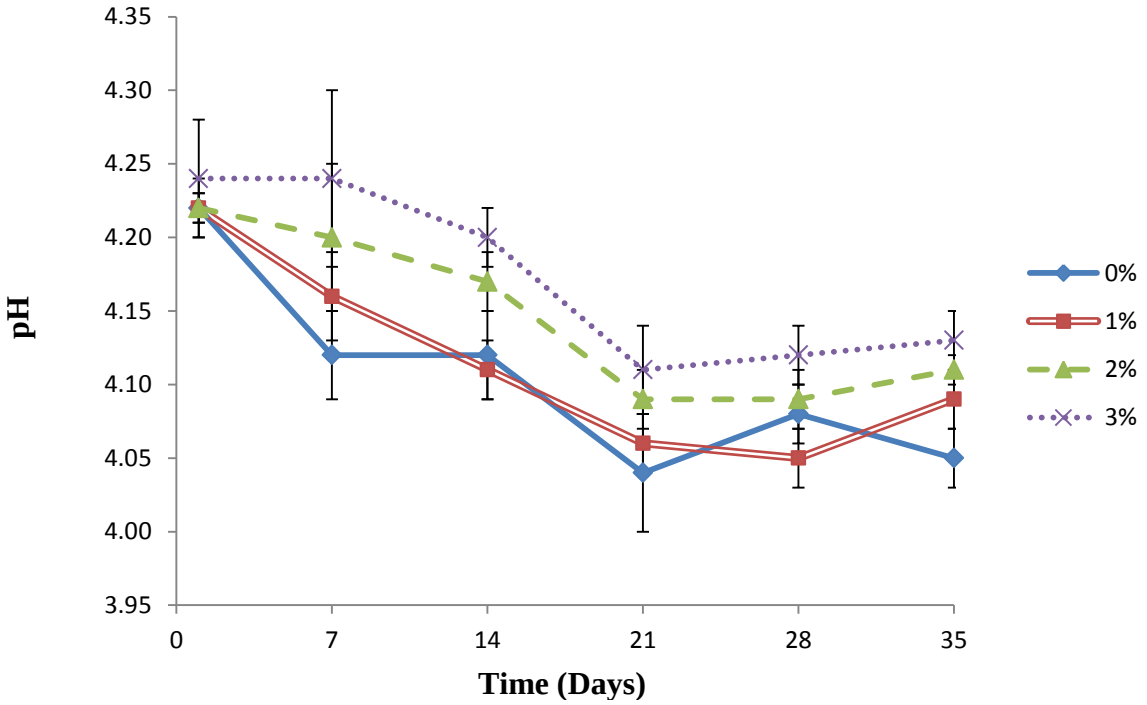

Figure 15. pH of yogurts as influenced by added whey protein isolate levels over storage period of 35 days.

Addition of 2 and 3% WPI showed higher pH values compared to 0 and 1% WPI (Table 23). Significant (P<0.05) pH decrease was observed at 7, 14, 21, 28 and 35 days of storage compared to day 1 (Table 24).

Marafon *et. al*. (2011) used WPC (0.5%) along with skim milk powder to increase protein of yogurt by 1g/100g of product and reported a drop in pH values in yogurts containing WPC and control over 28 days of storage. Significantly differences were not found by Marafon *et. al.* (2011) comparing control and addition of 0.5% WPC. This behavior is in accordance with this study where the addition of only 1% WPI did not have an effect on pH compared to control (P>0.05). On the contrary, addition of 2 and 3% WPI did have an effect on pH. According to Nadal *et. al*. (2010) addition of whey proteins can decrease the

acidification rate of the final product. According to Almeida *et. al*. (2009) a decrease in pH caused by post-fermentation acidification during storage is expected as result of the metabolic activity of *S. thermophilus* and *L. bulgaricus* which explain the changes of pH during storage.

Table 23. Least Square Means for pH of yogurts as influenced by added whey protein isolate concentrations.

Added Whey Protein Isolate (%)	pH
	LS Means
Control	4.10[c]
One	4.12[c]
Two	4.15[b]
Three	4.17[a]

[ab]LSMeans with different letter within the column are significantly different.

Table 24. Least Square Means for pH of yogurts as influenced by the storage period of 35 days.

Storage Period (Days)	pH
	LS Means
1	4.23[a]
7	4.18[b]
14	4.15[b]
21	4.07[c]
28	4.09[c]
35	4.10[c]

[ab] LSMeans with different letter within the column are significantly different.

3.9 Titratable Acidity (TA)

The Titratable Acidity (TA) of yogurts as influenced by WPI over storage of 35 days is shown in Figure 16. Treatment*day interaction effect, treatment effect and day effect were

significant (P<0.05) (Table 18).

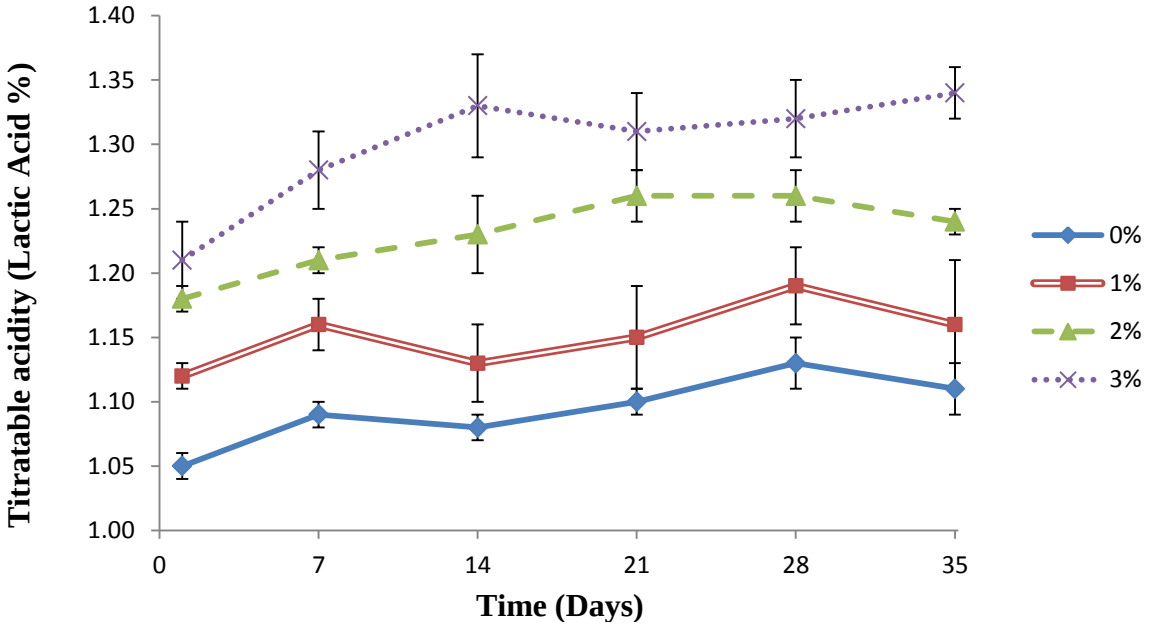

Figure 16. Titratable acidity (TA) of yogurts as influenced by added whey protein isolate levels over storage period of 35 days

Addition of 2 and 3% WPI showed significantly (P<0.05) higher titratable acidity (TA) values compared to control over the entire storage period of 35 days (Table 25). Use of 3% WPI showed significantly (P<0.05) higher titratable acidity (TA) values compared to 0, 1 and 2% WPI at 7, 14 and 35 days of storage (Table 25).

Heat processes during yogurt manufacture increase availability of amino acids and peptides for lactic acid bacteria growth (Dave and Shah, 1998b, Akalin *et. al*., 2007). As availability of free amino acids and peptides increased, the lactic acid production of yogurt culture bacteria increased as well (Akalin *et. al*., 2007). An increase in the lactic acid production does not necessarily drop pH values (Dave and Shah, 1998b). On contrary, addition of WPI significantly increased % of lactic acid in product (TA) while also increased pH values.

This behavior is also reported by Dave and Shah (1998b). They reported that addition of whey protein concentrates increased lactic acid production and pH compared to lower TA and pH values reported in control. Dave and Shah (1998b) explained that whey protein concentrate served as a buffer, controlling pH of the media.

Table 25. Least Square Means for Titratable Acidity (TA) of yogurts as influenced by added whey protein isolate concentrations over storage period of 35 days.

Added Whey Protein Isolate (%)	Titratable Acidity					
	Time (Days)					
	1	7	14	21	28	35
Control	1.05^{M}	1.09^{KLM}	1.08^{LM}	1.10^{JKLM}	1.13^{HIJKL}	1.11^{IJKLM}
One	1.12^{IJKLM}	1.16^{GHIJK}	1.13^{HIJKL}	1.15^{GHIJKL}	1.19^{EFGH}	1.16^{FGHIJ}
Two	1.18^{EFGHI}	1.21^{DEFG}	1.23^{CDEF}	1.26^{BCD}	1.26^{BCD}	1.24^{CDE}
Three	1.21^{DEFG}	1.28^{ABC}	1.33^{AB}	1.31^{AB}	1.32^{AB}	1.34^{A}

^{ABC} LSMeans with different letter within the table are significantly different.

3.10 Apparent Viscosity

The apparent viscosity of yogurts as influenced by WPI concentration over storage of 35 days is shown in Figure 17. Treatment*day interaction effect, treatment effect and day effect were significant (P<0.05) (Table 18).

Use of 1% WPI showed significantly (P<0.05) higher apparent viscosity compared to control at day 1 (Table 26). At 21 days of storage, 1% WPI showed significantly (P<0.05) higher apparent viscosity compared to 3% WPI (Table 26). In spite of the increased amount of added whey protein isolate, apparent viscosity was not affected at 35 days of storage (Table 26). Patocka *et. al.* (2006) added WPI to stirred yogurts after pasteurization and after

fermentation while WPI was added before pasteurization in this study. Patocka *et. al*. (2006) reported that addition of 1-3% whey protein isolate to prior manufactured stirred yogurt, did not have effect on the apparent viscosity of the product. According to Isleten and Karagul-Yuceer (2006) addition of 1% whey protein isolate increased apparent viscosity of nonfat yogurt which is in accordance with data obtained at 21 days of storage.

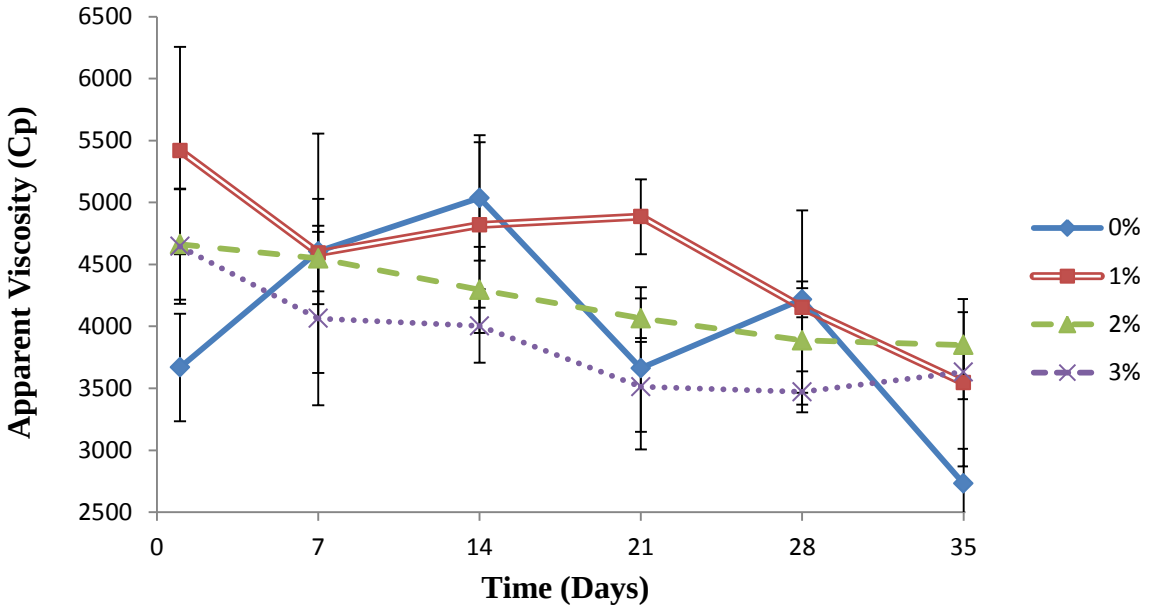

Figure 17. Apparent Viscosity of yogurts as influenced by added whey protein isolate levels over storage period of 35 days.

Table 26. Least Square Means for Apparent Viscosity of yogurts as influenced by added whey protein isolate concentrations.

Added WPI (%)	Apparent Viscosity					
	Time (Days)					
	1	7	14	21	28	35
Control	3668.8^{CDEF}	4604.5^{ABCDE}	5036.7^{AB}	3661.6^{CDEF}	4217.5^{ABCDE}	2731.1^{F}
One	5418.3^{A}	4590.6^{ABCDE}	4818.3^{ABCD}	4884.8^{ABC}	4153.0^{ABCDE}	3545.9^{DEF}
Two	4662.9^{ABCDE}	4547.8^{ABCDE}	4294.9^{ABCDE}	4064.6^{BCDE}	3886.2^{BCDEF}	3849.3^{BCDEF}
Three	4644.6^{ABCDE}	4063.2^{BCDE}	4003.3^{BCDEF}	3511.9^{EF}	3471.5^{EF}	3631.4^{CDEF}

^{AB} LSMeans with different letter within the column are significantly different.

3.11 Syneresis

The syneresis of yogurts as influenced by WPI concentration over storage of 35 days is shown in Figure 18. Treatment*day interaction effect, treatment effect and day effect were significant (P<0.05) (Table 18).

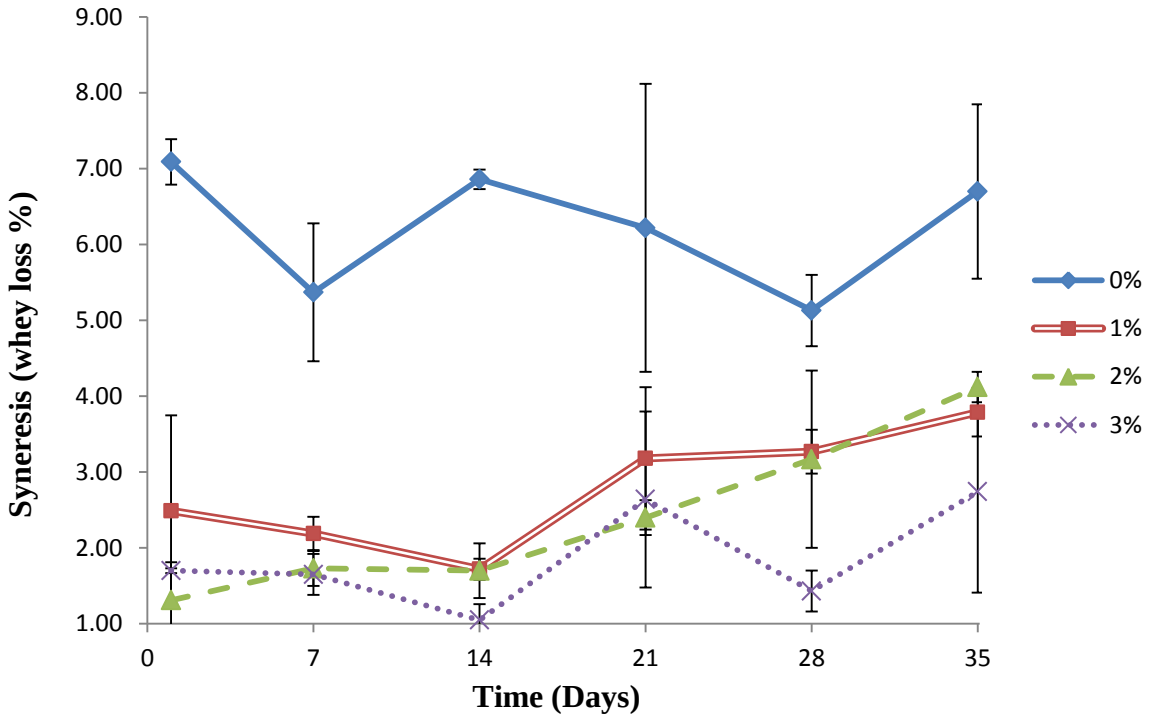

Figure 18. Syneresis (whey loss) of yogurts as influenced by added whey protein isolate levels over storage period of 35 days.

Addition of 1, 2 and 3% WPI showed significantly (P<0.05) lower syneresis values compared to control at 1, 7, 14, 21 and 35 days of storage (Table 27). In this study, upon addition of WPI, less syneresis was observed. Kücükcetin (2008) reported less syneresis of stirred yogurt at 1 day of storage when ratio of casein proteins to whey proteins was low. Li and Guo (2006) observed a reduction of syneresis (25%) when a solution containing 2.4% WPI was added to a goat yogurt mix. Marafon *et. al*. (2011) reported that addition of whey

protein concentrate (0.05%) created a more compact gel structure compared with no whey protein concentrate addition, thus creating a weaker gel.

Table 27. Least Square Means for Syneresis of yogurts as influenced by added whey protein isolate concentrations over storage period of 35 days.

Added Whey Protein Isolate (%)	Syneresis					
	Time (Days)					
	1	7	14	21	28	35
Control	7.09^{A}	5.37^{ABC}	6.86^{A}	6.22^{AB}	5.13^{ABCD}	6.70^{A}
One	2.49^{EFG}	2.19^{EFG}	1.72^{EFG}	3.18^{CDEFG}	3.27^{CDEFG}	3.79^{CDEF}
Two	1.31^{G}	1.73^{EFG}	1.70^{FG}	2.40^{EFG}	3.17^{CDEFG}	4.12^{BCDE}
Three	1.70^{FG}	1.65^{FG}	1.05^{G}	2.64^{EFG}	1.43^{FG}	2.74^{DEFG}

^{ABC} LSMeans with different letter within the table are significantly different.

3.12 Sensory Study

Means for all tested attributes (appearance, color, aroma, taste, thickness, graininess, and overall liking) are shown in Figure 19. Probabilities for fixed effect of sensory attributes are shown in Table 28. Appearance, Taste, Thickness, Graininess and Overall Liking were significant ($P<0.05$) while Color and Aroma were not significant ($P>0.05$) (Table 28).

Use of 1% WPI led to significantly ($P<0.05$) higher scores for appearance compared to 3% WPI (Table 29). Addition of WPI did not significantly ($P>0.05$) affect color and aroma of yogurts (Table 29). Use of 1% WPI showed significantly ($P<0.05$) higher taste scores compared to control (Table 29). Addition of 1% WPI led to significant ($P<0.05$) higher thickness scores compared to control, 2 and 3% WPI (Table 29). Control samples showed significantly lower scores for graininess ($P<0.05$) compared to all WPI samples (Table 29).

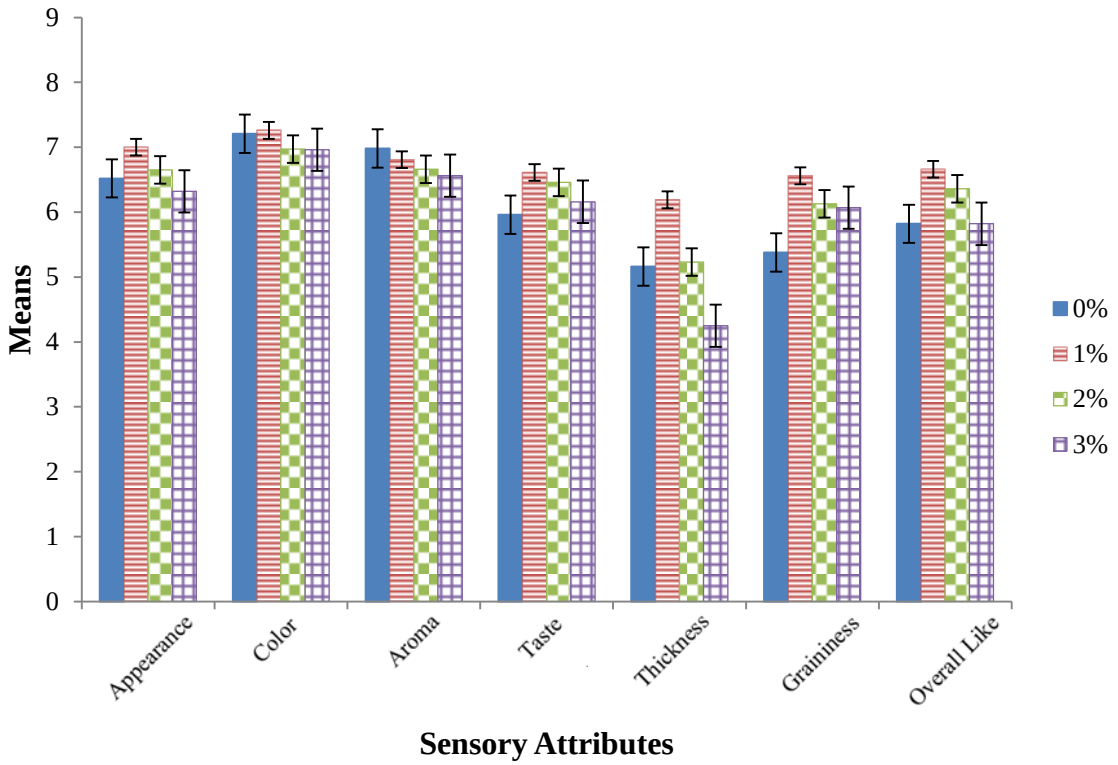

Figure 19. Means for sensory attributes of blueberry yogurt as influenced by added whey protein isolate.

Overall liking for yogurts containing 1% WPI led to significantly (P<0.05) higher scores compared to control and 3% WPI (Table 29). For thickness attribute, 3% WPI showed the lowest score which is in accordance with Patocka *et. al.* (2006) who reported lower apparent viscosity when higher amounts of WPI (>3%) was added to stirred yogurt. In contrast, addition of 1% WPI showed the highest score for thickness. Isleten and Karagul-Yuceer (2006) reported similar results when 1% WPI added to a yogurt mix, resulted in higher apparent viscosity compared with no whey protein isolate supplementation.

Table 28. Probability > F Value (Pr > F) for fixed effect of sensory attributes of yogurts containing 0, 1, 2 and 3% w/v added whey protein isolate.

Effect	Appearance	Color	Aroma	Taste	Thickness	Graininess	Overall Liking
Treatment	0.0076	0.245	0.1538	0.0406	<0.0001	<0.0001	<0.0001

Table 29. Means for sensory properties of yogurts as influenced by added whey protein isolate.

Added Whey Protein Isolate Concentration (%)	Sensory Attributes						
	Appearance	Color	Aroma	Taste	Thickness	Graininess	Overall Liking
Control	$6.52^{ab} \pm 1.44$	$7.21^{a} \pm 1.30$	$6.98^{a} \pm 1.32$	$5.96^{b} \pm 1.69$	$5.16^{b} \pm 1.71$	$5.38^{b} \pm 1.83$	$5.82^{b} \pm 1.55$
One	$7.00^{a} \pm 1.29$	$7.26^{a} \pm 1.18$	$6.81^{a} \pm 1.32$	$6.61^{a} \pm 1.65$	$6.19^{a} \pm 1.56$	$6.56^{a} \pm 1.60$	$6.66^{a} \pm 1.39$
Two	$6.65^{ab} \pm 1.48$	$6.97^{a} \pm 1.52$	$6.66^{a} \pm 1.52$	$6.46^{ab} \pm 1.94$	$5.23^{b} \pm 2.15$	$6.13^{a} \pm 1.88$	$6.36^{ab} \pm 1.77$
Three	$6.32^{b} \pm 1.64$	$6.96^{a} \pm 1.45$	$6.56^{a} \pm 1.55$	$6.16^{ab} \pm 1.92$	$4.25^{c} \pm 1.92$	$6.07^{a} \pm 1.75$	$5.82^{b} \pm 1.71$

[ab] Means with different letter within the column are significantly different.

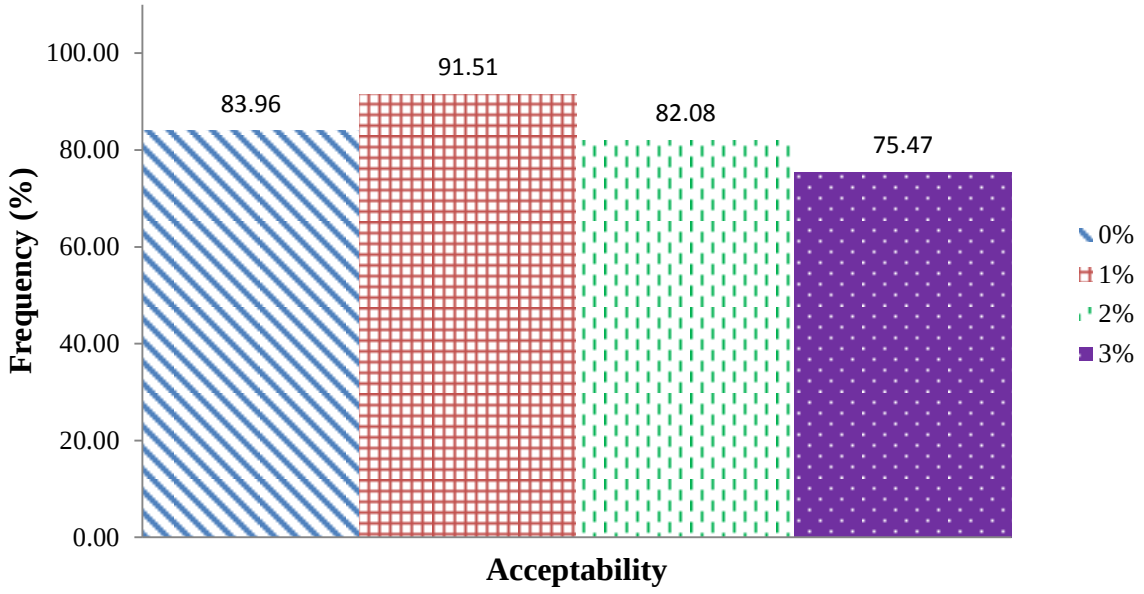

Figure 20. Frequency for acceptability of blueberry yogurt as influenced by whey protein isolate addition.

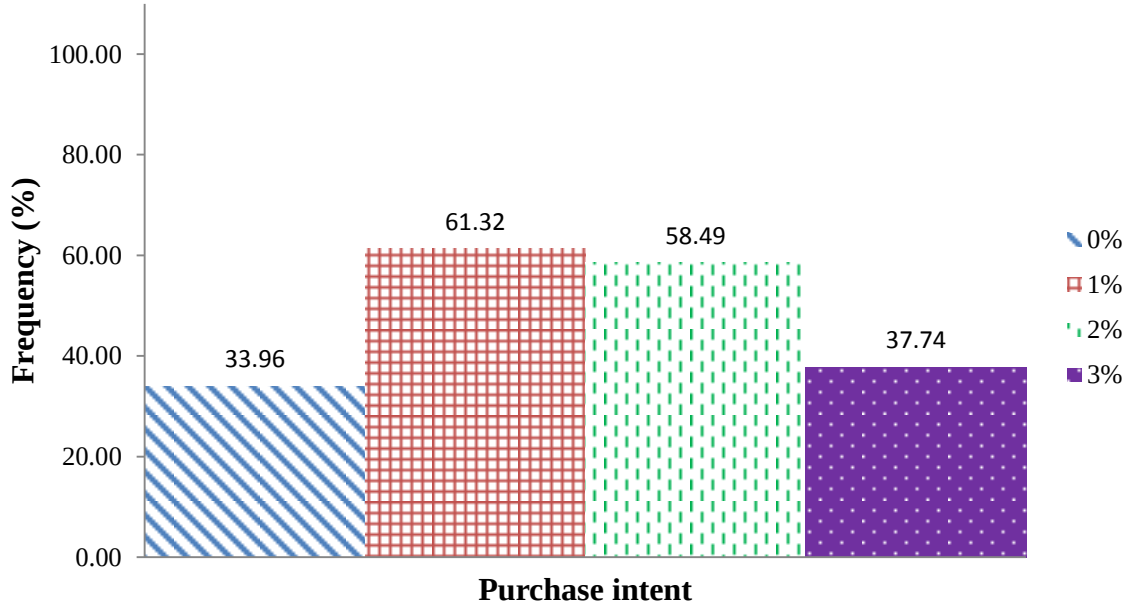

Figure 21. Frequency for purchase intent of blueberry yogurt as influenced by whey protein isolate addition.

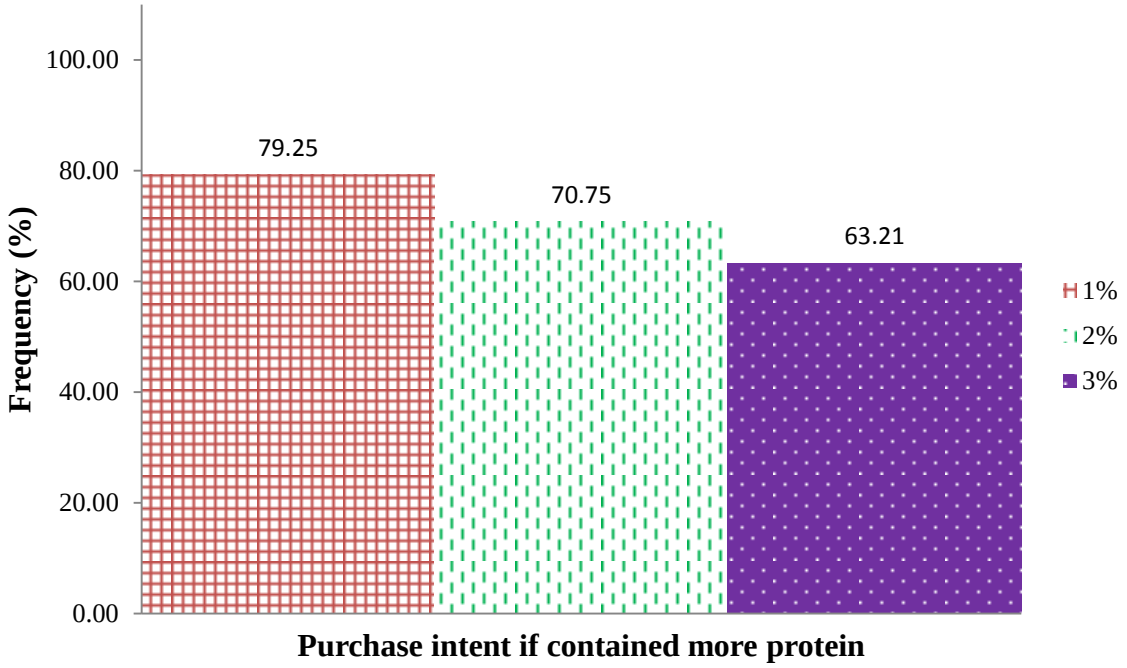

Figure 22. Frequency for purchase intent of blueberry yogurt as influenced by whey protein isolate addition, if panelist were informed about the extra protein and probiotic benefit of product.

Yogurt acceptability frequency values are shown in Figure 20. Nearly 92% of participants evaluated as acceptable yogurt containing 1% WPI compared to 75% of acceptability for 3% WPI. Acceptability of the product was slightly affected by addition of 2 and 3% WPI (82% and 75% respectively) compared to control (84%). According to Marafon *et. al*. (2011) fortification of yogurt with whey protein concentrate improved scores of acceptability and consistency and maintained this preferred characteristics over storage.

Yogurt purchase intent frequency values are shown in Figure 21. Addition of 1 and 2% WPI showed greater purchase intent values compared to control and 3% WPI. Yogurt containing 1% WPI led to higher purchase intent (61%) compared to control (34%).

Frequency for yogurt purchase intent if the participant was informed of health benefit of the product is shown in Figure 22. Purchase intent changed if the participant was informed about health benefits of the product. Purchase intent change from 37% to 63% in 3% WPI, from 58% to 70% in 2% WPI and from 61% to 79% in 1% WPI. Yogurt containing 1% WPI led to higher purchase intent compared with the rest.

CHAPTER 4: CONCLUSIONS

Results obtained from this study showed that addition of whey protein isolate (WPI) had a positive effect on probiotic properties of yogurt starter bacteria and yogurt characteristics. Use of 1, 2 and 3% WPI resulted in significantly higher acid and bile tolerance for pure cultures *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 for 120 minutes of exposure to acid and for 5 hours of exposure to bile. Use of 1, 2, and 3% WPI significantly increased growth of pure culture *Streptococcus thermophilus* ST-M5 at 24 hours of incubation compared to control. Use of 2 and 3% WPI resulted in significantly higher growth for pure *Lactobacillus bulgaricus* LB-12 from 24 to 60 hours of incubation compared to control and 1% WPI. Use of 1, 2 and 3% WPI significantly increased protease activity of both culture bacteria. Acid and bile tolerance of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 from manufactured fat free plain yogurt was evaluated at day 7 of storage. Use of 2% WPI significantly improved acid tolerance of *Streptococcus thermophilus* ST-M5 in yogurt. Bile tolerance of *Lactobacillus bulgaricus* LB-12 was significantly improved by use of 2 and 3% WPI compared to control over the 5 hours of bile exposure. Yogurts containing 2 and 3% WPI showed significantly higher pH and TA values compared to control. Apparent viscosity was not affected by WPI at 35 days of storage. Yogurts containing 1, 2 and 3% WPI resulted in significantly lower syneresis values (whey separation) compared to control over the entire storage period of 35 days. Addition of WPI had no effect on color and aroma of blueberry yogurt. Scores for taste, overall liking and acceptability were higher for 1 and 2% WPI. Addition of 1% WPI to yogurt contributed to higher scores for sensory thickness. Also the purchase intent

increased with the addition of 1 and 2% WPI. Overall, 1 or 2% WPI can be recommended in manufacture of higher whey protein yogurts.

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APPENDIX A. CONSENT FORM FOR CONSUMER STUDY

APPROVED BY
LSU AG CENTER
IRB AS HE 13-15a
ON 10/1/13

RESEARCH CONSENT FORM

I, _____, agree to participate in the research entitled "Influence of added whey protein isolate on some characteristics of yogurt culture bacteria and fat free yogurt" which is being conducted by the School of Animal Sciences at Louisiana State University, phone number (225)-578-4411.

I understand that participation is entirely voluntary and whether or not I participate will not affect how I am treated in my school. I can withdraw my consent at any time without penalty of loss of benefits to which I am otherwise entitled and have the results of the participation returned to me, removed from the experimental records, or destroyed. A total of at least 100 people will participate in this research. For this particular research, about a 10 minute participation will be required.

The following points have been explained to me:

1. In any case, it is my responsibility to report prior to participation to the investigators any allergies I may have.

2. The reason for the research is to gather information on the acceptance of yogurt cultured milk powder. The benefits that I may expect from it are a satisfaction that I have contributed to solution and evaluation of problems relating to such examinations.

3. The procedures are as follows: Coded samples of yogurts will be placed in front of me and I will evaluate them by normal standard methods and indicate my evaluation on score sheets. All procedures are standard methods as published by the American Society for Testing and Materials and the Sensory Evaluation Division of the Institute of Food Technologists.

4. Participation entails minimal risks: The only risk that can be envisioned is an allergic reaction to milk and lactose intolerance. However, because it is known to me beforehand what type of food to be tested, the situation can normally be avoided.

5. The results of this participation will be confidential and will not be released in any individual identifiable form without my prior consent unless required by law.

6. The investigator will answer any further questions about the research, either now or during the course of the project.

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators. In addition, I understand that research at Louisiana State University, which involves human participation, is carried out under the oversight of the Institutional Review Board. Questions or problems regarding these activities should be addressed to Michael Keenan, Chairman, Institutional Review Board, (225) 578 1708, mkeenan@agcenter.lsu.edu

Kayanush J. Aryana
Signature of Investigator

Date: 10/04/2013

Signature of Participant

Witness:

APPENDIX B. QUESTIONNAIRE FOR CONSUMER STUDY

Fat free blueberry yogurt: **Sample** ____

1. Please evaluate the product and mark the score [√] that best reflects your feeling about the product.
2. Between the samples, you are required to eat some crackers and drink water to clean your palate.

1. **OVERALL,** how would you rate the **overall appearance** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

2. **OVERALL,** how would you rate the **color** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

3. OVERALL, how would you rate the **aroma** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

4. **OVERALL,** how would you rate the **taste** this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

5. **OVERALL,** how would you rate the **thickness** this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

6. **OVERALL,** how would you rate the **graininess** this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

7. **OVERALL,** how do you **"LIKE"** this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

8. Is this product **ACCEPTABLE**? YES [ ] NO [ ]

9. Would you **BUY** this product if it were commercially available? YES [ ] NO [ ]

HEALTH BENEFIT:

High protein yogurt = Health Benefits

10. Would you **BUY** this product knowing it is high in protein? YES [ ] NO [ ]

VITA

Luis Alfonso Vargas Lopez was born in Chiriqui, Panama, on February, 1988. In fall 2009 he received his Bachelor of Science degree in Food Science and Technology from Escuela Agrícola Panamericana, Zamorano University in Tegucigalpa, Honduras. During spring 2009 he participated in an internship at Melo Group Panama, where he worked as a quality assurance supervisor in a facility of fast food pre-mixes. Before becoming a graduate student in the School of Animal Sciences/Dairy Science Division at Louisiana State University in the fall of 2011, he worked as Area Manager and Delivery Project Coordinator for Melo Group in Panama city, Panama. At this position, he had the opportunity to create a delivery system for a fast food restaurant franchise, support workers through personalized training in standard procedures and food safety and manage operations of restaurants in different areas of Panama city, Panama. While a graduate student at Louisiana State University, he participated in leading positions at various student clubs such as LSU Food Science Club and Zamorano Agricultural Society (ZAS). Currently, he is a candidate for Master´s degree of Dairy Foods Technology from Louisiana State University and Agricultural Mechanical College in December 2013.